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An Examination of Marine Snow as a Pathway for Mercury Uptake in
the Blue Mussel *Mytilus edulis*

Veronica Lee Ortiz

B.S. Chemistry, Environmental Concentration, Roger Williams University, 2009

A thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

At the

University of Connecticut

2013

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2013

APPROVAL PAGE

Masters of Science Thesis

An Examination of Marine Snow as a Pathway for Mercury Uptake in the Blue Mussel *Mytilus edulis*

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2013

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Chapter 1-Introduction

Mercury

Mercury (Hg) is a heavy metal known for its unique physical characteristics and potent neurotoxicity especially in the organic forms (mostly as methylmercury [CH_3Hg]). In the pure state, Hg is a dense, volatile, silver-white liquid at ambient temperatures. However, in the environment Hg is present in various forms including: elemental mercury (Hg^0), ionic and inorganic mercury species ($\text{Hg}(\text{II})$, $\text{HgCl}(\text{I})$, HgCl_2^0), and organic species (methylmercury, CH_3Hg and dimethylmercury, $(\text{CH}_3)_2\text{Hg}$). Uses and releases during industrial processes such as artisanal gold mining, coal burning, and waste incineration have widely distributed the toxic element (mostly in the inorganic form) throughout the environment, primarily into the atmosphere (Pirrone et al., 2010; Fitzgerald et al., 2007; Clarkson and Magos, 2006; Morel et al., 1998). Mercury has a relatively long residence time in the atmosphere of 6 to 12 months (Morel et al., 1998) resulting in its global distribution and contamination. Approximately 16 Mmol yr^{-1} of Hg is deposited to aquatic environments resulting in Hg being a ubiquitous metal in both fresh water and marine ecosystems (Figure 1) (Pirrone et al., 2010, Fitzgerald et al., 2007). This high abundance of Hg in aquatic environments is a public health concern due to the neurotoxic properties, especially CH_3Hg .

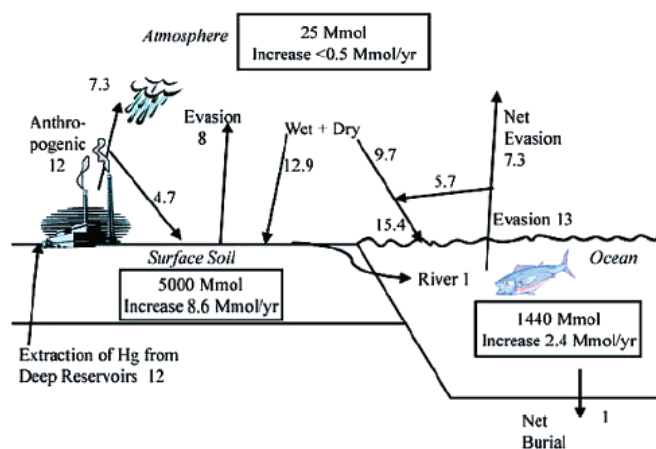


Figure 1-Mercury mass balance (post-industrial period) indicating fluxes in Mmol yr⁻¹. Bold boxes highlight fluxes of anthropogenic emissions and deposition of Hg into terrestrial and marine sinks and reservoirs (Diagram by Fitzgerald et al., 2007).

The potential toxicity of different Hg compounds is attributed to either its lipophilicity (Hg⁰) or its sulfur-binding affinity (CH₃Hg and Hg(II)). Although elemental mercury (Hg⁰) has been observed to penetrate the lipid bilayer of cells (Veltman et al., 2008; Clarkson and Magos, 2006; Blackmore and Wang, 2004; Lawrence and Mason, 2001; Ullrich et al., 2001), it does not bioaccumulate unless it is oxidized in the tissues. In contrast, CH₃Hg can bioaccumulate through various mechanisms, but primarily by strongly binding to sulfur-rich proteins (Clarkson and Magos, 2006).

The toxic properties of CH₃Hg are a major public health concern particularly for children and expecting mothers. In expecting mothers, CH₃Hg can have impacts on the fetus because it can cross the placenta and be taken up by the developing child (Clarkson and Magos, 2006; Clarkson et al., 2003; Ullrich et al., 2001; Aschner and Aschner, 1990). CH₃Hg can react to form charge-neutral forms of Hg that can diffuse into cells and cross the blood brain barrier (BBB) or placenta leading to serious neurological damage and disrupted mental development (US EPA, 2001; Morel et al., 1998). Women, especially pregnant women, and children are strongly encouraged to include fish in their diet to incorporate omega-3 fatty acids, which are linked to healthy brain development (Sunderland, 2007). Unfortunately, many consumers are unaware of the need to ingest lower trophic level fish which contain

healthy omega-3 fatty acids but have lower concentrations of CH_3Hg than higher trophic level fishes. Diets consisting of large quantities of high trophic level fish can put consumers at risk of CH_3Hg poisoning, and children at risk of mental retardation as exposure is determined by both the CH_3Hg content of the fish and the amount consumed. When humans eat large quantities of fish with elevated Hg content, they also accumulate a high concentration of CH_3Hg in their tissues (Clarkson and Magos, 2006). The incidences at Minimata Bay and Niigata, Japan are two of the most well-known Hg poisonings caused by CH_3Hg -contaminated food (fish and shellfish) that was bioaccumulated and biomagnified through the food chain from industrial pollution. The poisonings caused neurological damage including loss of sensation, slurred speech, diminution of vision, and death. Thereafter, serious to lethal CH_3Hg poisoning has been referred to as Minimata disease (Clarkson and Magos, 2006; Takizawa, 1979). Many coastal populations rely on seafood as a primary source of protein, particularly Arctic and island nations; therefore, such populations are vulnerable to increased Hg inputs and its conversion to CH_3Hg in coastal environments.

Bioaccumulation is defined as the increase of any toxic contaminant over multiple trophic levels (Kimbrough et al., 2008, O'Connor, 2002). As an example, CH_3Hg is thought to passively diffuse across the membrane of phytoplankton as neutral complexes (Mason et al, 1995). The resulting phytoplankton cell concentration of CH_3Hg is, therefore, related to the concentration and speciation of CH_3Hg in the medium. Next, larger primary consumers prey upon the phytoplankton and subsequently increase their CH_3Hg body burden. This trend continues up the food chain to top predators such as sharks, swordfish, whales, birds of prey, and humans. For instance, reef fish have an average of $49 \text{ pmol g}^{-1} \text{ CH}_3\text{Hg}$ in their tissues, while sharks have an average of $19 \text{ nmol g}^{-1} \text{ CH}_3\text{Hg}$, while the surrounding water has a concentration of approximately 4.9 pM . Therefore, by the time the CH_3Hg accumulates in the shark tissue the concentration has increased by a million-fold (Clarkson and Magos, 2006; Fitzgerald et al., 1991). It is important to note that as the concentration of CH_3Hg increases over trophic levels, so does the percentage of CH_3Hg relative to Hg(II) as it bioaccumulates through the food web (Lawrence and Mason, 2001).

Legacy pollution and pollution events (i.e., chemical spills, oil spills, etc) are of greatest concern in coastal ecosystems because of the extensive chemical and biological reactions that occur depending upon salinity, temperature, oxygen, pH, and alkalinity. For example, Hg will form different complexes in freshwater ($\text{Hg}(\text{OH})_2$) than in saltwater (HgCl_2). Some metals experience reactions that render them labile and biologically available (i.e. $\text{Hg}(\text{II}) \rightarrow \text{CH}_3\text{Hg}$) while other metals are converted to inert compounds (i.e., $\text{Fe}(\text{II}) \rightarrow \text{Fe}(\text{OH})_3(\text{s})$). Hg is primarily introduced in the oxidized form, $\text{Hg}(\text{II})$, into coastal systems via atmospheric deposition, inputs from the watershed, and exchange with the open ocean (see below; Figure 2) (Pirrone et al., 2010; Fitzgerald et al., 1991). Once deposited into the water column $\text{Hg}(\text{II})$ can be reduced to Hg^0 and evaded back into the atmosphere, scavenged by settling particulates in the water column and buried in sediments, or methylated to CH_3Hg by bacteria (sulfur- and iron-reducing bacteria) (Figure 2) (Fitzgerald et al., 2007; Fitzgerald and Mason, 1997). Settling particulates in the water column act as vectors for the transport of metals and nutrients from the euphotic zone to the benthos in coastal systems (de la Rocha, 2006; Simon et al., 2002; Alldredge and Silver, 1988). These particulates increase the bioavailability of nutrients (NO_3^- , PO_4^{3-}), but may also increase the bioavailability and transport of trace metals.

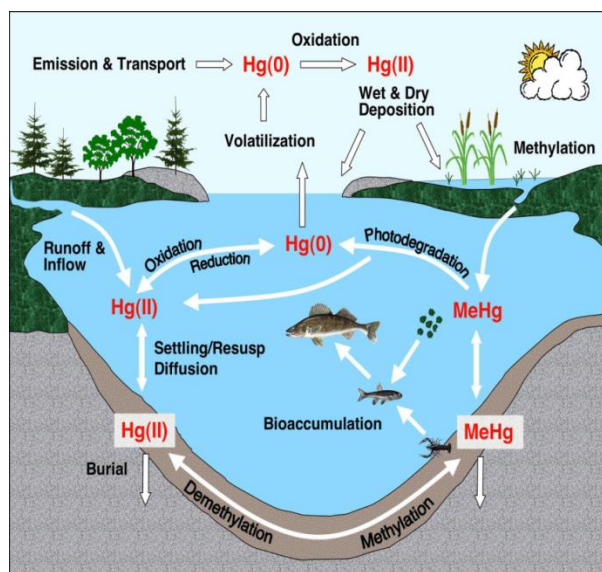


Figure 2-Mercury cycling in aquatic environments and bioaccumulation into the food web (Diagram by Engstrom, 2007).

Marine Snow

Settling particulates in the water column are referred in the literature as marine snow ($>100\mu\text{m}$), flocs, or marine aggregates. For the remainder of this paper, I will refer to large, visible settling particulates as marine snow. In my experiments, I visually isolated and collected these large particulates from other smaller particulates. Marine snow is composed of transparent exopolymer particulates (TEP), living organisms and their detritus, inorganic particulates, fecal pellets, and organic matter (humic and fulvic acids). These materials coagulate as they settle through the water column as a result of collisions caused by Brownian motion, physical shear, and differential settling (Kach and Ward, 2008; Lyons, 2008; Li et al., 2008; Heinonen et al., 2007; de la Rocha, 2006; Jackson and Burd, 1998; Alldredge et al., 1993; Kiørboe et al., 1990; Alldredge and Silver, 1988). These macroscopic particulates (particles $3\text{-}100+\mu\text{m}$) are important not only to the cycling of nutrients from the euphotic zone to the benthic zone, but also to that of trace metals (de la Rocha, 2006; Simon et al., 2002; Alldredge and Silver, 1988).

The chemical environment of marine snow is still unresolved. However, some research has shown that particulate aggregation can create “microzones” in the water column with chemical characteristics that markedly differ from the surrounding water column. Alldredge and Cohen (1987) were the first to present evidence of oxygen and pH gradients that occur within a 1mm diameter from the outside to the inside of marine snow. Oxygen concentrations measured at the particle surface were higher than the ambient seawater indicating photosynthetic activity. Once inside the marine snow oxygen concentrations sharply decreased suggesting that anaerobic conditions may be maintained within marine snow (Alldredge and Cohen, 1987). Alldredge and Silver (1988) suggest that as marine snow forms, organic-binding substances and biologically labile gasses may become entrained in the forming particulate. Combining possible anaerobic conditions with organic-binding substances and biologically available gasses may create suitable conditions for Hg methylation within marine snow. Additionally, since Hg is highly particle reactive, it can be assumed that CH_3Hg and Hg(II) will be readily incorporated into marine snow as it forms and settles through the water column, although it has not been researched

(Kim et al., 2008; Fitzgerald et al., 2007; Lawrence and Mason, 2001). Incorporation of CH₃Hg and Hg(II) into marine snow introduces an additional route of uptake, besides those typically considered, into the food web as various organisms (i.e., zooplankton and bivalve molluscs) can ingest these macroscopic particulates (Dilling et al., 1998; Kach and Ward, 2008; respectively).

Bivalves

This transport of trace metals to the benthos via settling particulates may increase the uptake of toxic metals by benthic biota including suspension and deposit feeding bivalves that may otherwise not have been exposed. Suspension and deposit feeding bivalves, such as mussels and clams, can assimilate and accumulate CH₃Hg as they filter particulates from the water and process sediments for microscopic organisms and organic particulates (Kimbrough et al., 2008; Wang and Fisher, 1996; Roesijadi, 1982). These benthic organisms are then consumed by larger pelagic predators or humans thereby introducing CH₃Hg and Hg(II) to higher trophic levels. Bioaccumulation studies have been conducted utilizing various bivalve species to determine their uptake and assimilation of various toxins from water, food, or sediments (Blackmore and Wang, 2004; Griscom and Fisher, 2004; Griscom et al., 2000; Boening, 1999; Wang et al., 1996; Wang and Fisher, 1996; Wang et al., 1995), but virtually no research has been conducted concerning the uptake of toxins from marine snow settling in the water column.

The research herein primarily concerns the use of the blue mussel, *Mytilus edulis*, as a bioaccumulation test subject. The blue mussel is an abundant semi-sessile intertidal organism that feeds primarily on suspended particles in the water column including plankton, detritus, and marine snow (Newell and Moran, 1989). These organisms are able to filter large volumes of water and concentrate particulates in their gut (Roesijadi, 1996; Livingstone and Pipe, 1992; Widdows and Donkin, 1992), thereby simultaneously concentrating toxins bound to those particulates (Wang and Fisher, 1996). The blue mussel has been observed to bioconcentrate environmental contaminants by a factor of 10 to 10⁵ relative to the water concentration, especially organometallic and heavy metal toxins (Byrne and

O'Halloran, 2001; Widdows and Donkin, 1992; Newell and Moran, 1989). Mussels are easy to culture and maintain in laboratory environments making them ideal for environmental monitoring and experimental studies (Byrne and O'Halloran, 2001; Boening et al., 1999; Inza et al., 1997). A primary example of a widespread environmental biomonitoring study is the Mussel Watch program. The Mussel Watch program consisted of annually collected data (1986 to 2005) from 300 sites along the coasts of the United States. Mussels and oysters were analyzed for approximately 140 different analytes of organic and metal contaminants (Kimbrough et al., 2008; O'Connor, 2002; Goldberg et al., 1978). This nationwide study demonstrated how mussels and oysters serve as sentinel species of pollution and can reflect changes in pollution over time and space.

Most bivalves have a capture efficiency of >90% for particles with a diameter >6 μm . However, their capture efficiency begins to exponentially decrease as particle size decreases below 6 μm (Ward and Shumway, 2004; Newell and Moran, 1989; Møhlenberg and Riisgård, 1978). The limitation of capture efficiency by particle size implies that bivalves are not able to capture dissolved or colloidal materials, thus making them unlikely to assimilate dissolved or colloidal CH_3Hg or Hg(II) fractions. However, the organic composition of marine snow may promote the incorporation of dissolved metals making otherwise unavailable CH_3Hg or Hg(II) bioavailable to suspension feeding organisms in intertidal and benthic zones.

This study combined the use of the commonly used sentinel species, *Mytilus edulis*, and laboratory-produced marine snow to test the potential uptake and assimilation of CH_3Hg and Hg(II) via marine snow, which has not been studied in detail. First, I wanted to determine if it was possible to incorporate both dissolved forms of Hg (i.e., CH_3Hg and Hg(II)) into laboratory produced marine snow and if this would make them bioavailable. Next, I determined if feeding Hg-contaminated marine snow to blue mussels over a 2-hr period would result in an increased tissue concentration compared to mussels exposed to uncontaminated marine snow and to mussels in seawater without marine snow production. In conclusion, my first stated hypothesis was that CH_3Hg and Hg(II) would be incorporated into marine snow. My second hypothesis was that mussels would accumulate CH_3Hg and Hg(II) in their digestive

glands from contaminated marine snow (Inza et al., 1997; Widdows and Donkin, 1992). The approach outlined above allowed the examination of these hypotheses using laboratory produced marine snow and Hg exposure studies.

Chapter 2 – Quantifying incorporation of methylmercury (CH_3Hg) and inorganic mercury (Hg(II)) into marine snow over time and partitioning of Hg in individual mussel tissues

Introduction

Incorporation of CH₃Hg and HgT into marine snow

As previously stated, marine snow is composed of transparent exopolymer particulates (TEP), living organisms, inorganic particulates, fecal pellets, and organic matter (humic and fulvic acids) (Alldredge et al., 1993; Jackson and Burd, 1998; de la Rocha, 2006; Heinonen et al., 2007; Kach and Ward, 2008). It is known that CH₃Hg and Hg(II) strongly partition to suspended particles likely through their association with sulfur-rich proteins and thiol-groups in humic acids (Haitzer et al., 2003; Coquery et al., 1997; Decho and Luoma, 1994). Once CH₃Hg and Hg(II) bind to these settling particulates (e.g., marine snow) they can then potentially be ingested by suspension feeding bivalves resulting in tissue accumulation when accumulation may not have occurred in the absence of marine snow.

Current research has investigated partition coefficients ($\log K_d$) between Hg (both CH₃Hg and Hg(II)) and organic sediments, dissolved organic material, and colloidal material (Lawrence and Mason, 2001; Coquery et al., 1997; Stordal et al., 1996; respectively). Each study observed high $\log K_d$ values ranging from 10^5 to 10^6 , indicating a strong binding relationship between Hg and organic matter, but little research has been conducted specifically regarding incorporation of Hg into marine snow. In addition, no research has examined whether incorporation into marine snow would be greater than incorporation into smaller particulates. The results of previous studies indicated adsorption to high-organic particulates increased scavenging of Hg from solution and a decrease in bioavailability to biota (Lawrence and Mason, 2001). Since marine snow is composed of living and non-living material, the bioavailability of Hg from marine snow may be very different than from other studied particulates.

The primary purpose of this experiment was to determine the potential to incorporate CH₃Hg and Hg(II) into marine snow. In addition to determining whether or not incorporation of CH₃Hg and Hg(II) into marine snow occurred, I determined the time it would take to incorporate the maximum amount of CH₃Hg and Hg(II) into the marine snow. Such knowledge would help in designing feeding experiments. The results from this experiment could also increase the understanding of the potential for Hg

incorporation into environmental marine snow which would be useful for large-scale mass balance modeling.

Bioaccumulation of CH₃Hg and Hg(II) in mussel tissues from the uptake of Hg-contaminated marine snow

Extensive research has been conducted and published regarding bioaccumulation patterns using the blue mussel because of its stamina and endurance in a laboratory environment and its ecological and economic importance (Byrne and O'Halloran, 2001; Widdows and Donkin, 1992; Newell and Moran, 1989). Toxicity tests have been conducted on mussels to determine LC₅₀ and EC₅₀ using phytoplankton exposed to dissolved Hg (Sauvé et al., 2002), whereas others have used phytoplankton or sediments labeled with Hg radioisotopes to trace the fate of Hg in the mussels (Wang et al., 2004; Griscom et al., 2004; Gagnon and Fisher, 1997). Sauvé et al. (2002) determined that ca. 1.3×10^{-5} M CH₃HgCl and ca. 7.2×10^{-5} M HgCl₂ were required to induce a decrease in hemocyte phagocytosis in 50% of the population (i.e., EC₅₀). Griscom et al. (2004), Gagnon and Fisher (1997), Fisher et al. (1996), and Wang et al. (2004) observed tissue distribution and assimilation efficiencies of various heavy metals after rapid-pulse exposures to sediment and phytoplankton, respectively. Each found the highest accumulation of CH₃Hg occurred in the digestive gland, visceral mass, and the proteinaceous foot and adductor muscles, while the highest accumulation of Hg(II) was in the gills and mantle similar to results presented by Livingston and Pipe (1992) and King and Davies (1987).

The goal of this project was to determine and quantify if and how much bioaccumulation of CH₃Hg and Hg(II) occurs in the blue mussel after an acute exposure to contaminated marine snow. To examine not only uptake, but also tissue partitioning this study design focused on the analysis of tissues that would most likely accumulate CH₃Hg and Hg(II) including: the gill, the mantle, the digestive gland, and the foot and adductor muscles (Reinfelder et al., 1998; Inza et al., 1997; Gagnon and Fisher, 1997; Wang et al., 1996; Wang and Fisher, 1996; Roesijadi, 1982). Gills are considered the most important

tissue for soluble uptake (labile Hg(II) and CH₃Hg), whereas the digestive gland is the most important for particulate uptake. Lipid-soluble metals are able to cross the thin cell layers of the gill tissue while phagocytosis in the digestive gland is the primary mechanism of uptake for particulate bound metals (Livingstone and Pipe, 1992; King and Davies, 1987). Haemolymph cells are used in the intracellular process of digestion (i.e., phagocytosis). When these cell phagocytose a contaminant or xenobiotic they incorporate that metal into the cellular materials and thereby introduce the xenobiotic to the entire organism (Sauvé et al., 2002). The remaining tissues (the foot and adductor muscles) reflect physiological repartitioning and redistribution of CH₃Hg and Hg(II) as more of the protein-rich tissues grow. Lastly, metals and xenobiotics can accumulate in the haemolymph and cause severely damaging immune suppression (Sauvé et al., 2002; Byrne and O'Halloran, 2001).

I hypothesized that the mussels would ingest and accumulate CH₃Hg and Hg(II) from contaminated marine snow. I expected the greatest increase in concentration to occur in the digestive gland because I did not expect the mussels to process and partition CH₃Hg and Hg(II) into other tissues within the two hour feeding assay. Results from this experiment would therefore show whether or not marine snow increases the bioavailability of CH₃Hg and Hg(II). In the following procedures Hg(II) is referred to as HgT (total mercury) as these two fractions are essentially equivalent given the experimental design.

Materials and Methods

Incorporation of CH₃Hg and HgT into marine snow

All materials were trace-metal cleaned before conducting experiments. Materials included: 1-L glass rolling jars, 0.2-µm polycarbonate filters, 15-mL polyethylene Falcon tubes, 2-mm glass frit filters, graduated filter holders, and 9" Pasteur pipets. All of these materials were cleaned in 5% nitric acid (HNO₃) for a minimum of three days. After soaking, they were rinsed three times with Millipore water and soaked in 2% HNO₃ for at least three days, then rinsed and dried in a laminar flow hood.

Marine snow was produced using the roller-table method described by Shanks and Edmondson (1989). Seawater was collected from UCONN Avery Point beach (41° 18.954 N, 72° 03.588 W) and passed through a 210- μ m mesh to remove large particles and zooplankton. The water was characterized by measuring the pH, temperature, salinity, and alkalinity. Subsamples of the seawater were filtered through pre-ashed 25-mm GF/F filters into pre-ashed scintillation vials for DOC (dissolved organic carbon) analysis. Additional aliquots of seawater were filtered through pre-ashed 25 mm GF/F filters which were collected for CHNS (carbon, hydrogen, nitrogen, and sulfur) analysis. The remaining 210- μ m sieved seawater was poured into clean 1-L glass jars.

Experiments to examine the incorporation of CH_3Hg and HgT into marine snow were conducted at separate times; i.e., the incorporation of CH_3Hg was examined during a separate experiment from HgT incorporation. Each incorporation experiment included control jars filled with the sieved seawater without Hg addition. Duplicate jars with and without Hg were placed on a roller table to produce marine snow (referred to as the “rolled” treatment), while an identical set of jars were left on a stationary surface such that marine snow formation was not enhanced (referred to as the “unrolled” treatment). The jars for the CH_3Hg incorporation experiment were spiked with 0.25 nM CH_3Hg standard. The jars for the HgT experiment were spiked with 4.9 nM HgT standard. Rolled jars in both experiments were rolled at 10 rpm in a temperature controlled environmental chamber set to 18 °C with a light/dark cycle for 10 days (Figure 3). Duplicate jars (rolled and unrolled) were removed from the rolling table every 48 hrs after the fourth day in the CH_3Hg experiment and after the third day in the HgT experiment.



Figure 3-Glass jars filled with sieved seawater spiked with 0.25 nM CH₃Hg or 4.9 nM HgT on rolling tables in an environmental chamber to produce marine snow.

Once removed from the rolling table, rolled jars were allowed to settle for 15 minutes before any visible marine snow was collected using a clean disposable pipet. Unrolled jars were removed from the stationary table and gently inverted three times and allowed to settle for 15 minutes. Water was randomly sampled from the bottom of the unrolled jars such that the clean pipet did not come in direct contact with the glass bottom. Collected marine snow was gently filtered through 0.2- μ m polycarbonate filters under low vacuum and then rinsed with an ammonium formate (NH₄COOH) solution. The NH₄COOH solution was prepared at the same concentration as the measured salinity of the collected water. This slightly volatile solution was used to exchange any salts out of the marine snow to yield a more accurate weight. The same method was used for the material collected from the unrolled jars. All filters were lyophilized for 72 hrs to obtain a dry sample weight. Aliquots of water from each jar were collected after marine snow (in rolled jars) or settled materials (in unrolled jars) were removed and filtered. The water samples were preserved in 0.5% hydrochloric acid (Fisher Trace-Metal Grade) and refrigerated.

For HgT analysis, the dried marine snow from the rolled jars and the dried settled particulates from the unrolled jars were separately digested in 5-mL of 4 M HNO₃ (Fisher Trace-Metal Grade) and 0.2% bromine monochloride (BrCl; Baker Potassium Bromide, Potassium Bromate, Fisher Trace-Metal

Grade Hydrochloric Acid) and left to incubate in a warm-water bath at 60 °C overnight. The resulting solution was analyzed using cold vapor atomic fluorescent spectroscopy (CVAFS). BrCl is used as an oxidizer because it is an unstable molecule that is highly reactive. Both halogen atoms will extract one electron from other molecules to obtain a noble gas electron configuration filling their valence orbitals. The oxidative properties of BrCl break bonds between Hg and organic matter by oxidizing the organic matter and thus freeing Hg(II) into solution. BrCl will also decompose CH₃Hg and oxidize any Hg⁰ present. Prior to analysis, 0.1% of 12% hydroxylamine hydrochloride (w/v) was added to the digest as a pre-reductase to reduce remaining reactive halogens in the solution. A 1-mL aliquot of this final digest solution was spiked into nitrogen-purged reagent seawater followed by 100-μL of stannous chloride (SnCl₂, Fisher, safe for Hg determination) to reduce the free Hg(II) to Hg⁰. The solution was then purged in a glass bubbler under nitrogen gas for 10 minutes onto a gold trap (Hammerschmidt and Fitzgerald, 2006; Hammerschmidt and Fitzgerald, 2005; EPA method 1631; Bloom and Crecelius, 1983) (Figure 4). The gold trap was burned at ca. 450 °C, under argon, releasing any trapped Hg from the gold column into a Tekran fluorescence detector. Peaks were detected and integrated using Peak388 software and resulting peak areas were used to calculate measured Hg concentrations. After every 5 samples, an aqueous blank and gas standard check were completed to ensure a steady blank value, evaluate sample trap recovery, and indicate any instrument drift. A lab duplicate and an aqueous standard check were run after every 10 samples per bubbler to ensure quality control. Sample volume was limited so matrix spikes were unable to be completed for this study. Standard reference materials (SRM) were digested and analyzed in the same method as the samples to serve as quality controls. Tort-2 (National Research Council Canada certified lobster hepatopancreas reference material; certified range, 1.05-1.65 nmol g⁻¹ HgT) and Mess-3 (National Research Council Canada certified marine sediment reference material; certified range, 0.414-0.494 nmol g⁻¹ Hg(II)) were the SRMs used for HgT analysis. Tort-2 SRM (certified range, 0.691-0.821 nmol g⁻¹ CH₃Hg) was used for CH₃Hg analysis. SRM recoveries were accepted within the range of the certified value. Relative percent differences (RPD) between analytical duplicates were accepted within

24% coincidence for HgT analysis and 35% for CH₃Hg analysis (EPA Method 1631 and 1630, respectively).

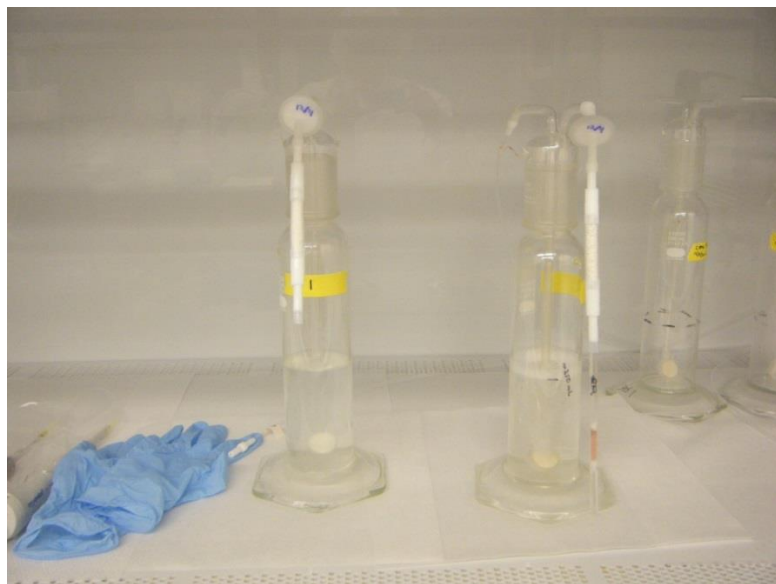


Figure 4-Bubblers used for CVAFS analysis. Bubbler on right was actively being purged with Ar gas to capture Hg⁰ onto a trap attached to the end of a soda lime trap used to trap any residual water vapor before the Hg trap.

Methylmercury analysis was completed using gas chromatograph (GC) couple with CVAFS. Collected marine snow (from rolled jars) and settled particulates (from unrolled jars) were distilled in heated reaction vessels with 200- μ l potassium chloride (KCl) and 50% sulfuric acid (H₂SO₄). Potassium chloride is used to form chloride complexes with any CH₃Hg and Hg(II) present which have a boiling point similar to water (ca. 110 °C). The H₂SO₄ is used to decrease the pH of the solution (which increases the chloride complexation; Figure 5) and to break down organic matter that may be bound to CH₃Hg and Hg(II) in the solution. Boiling the solution releases both CH₃HgCl and HgCl₂ complexes from the liquid matrix in the form of vapor which is collected in cooled receiving vessels connected to the distillation vessel (Figure 6).

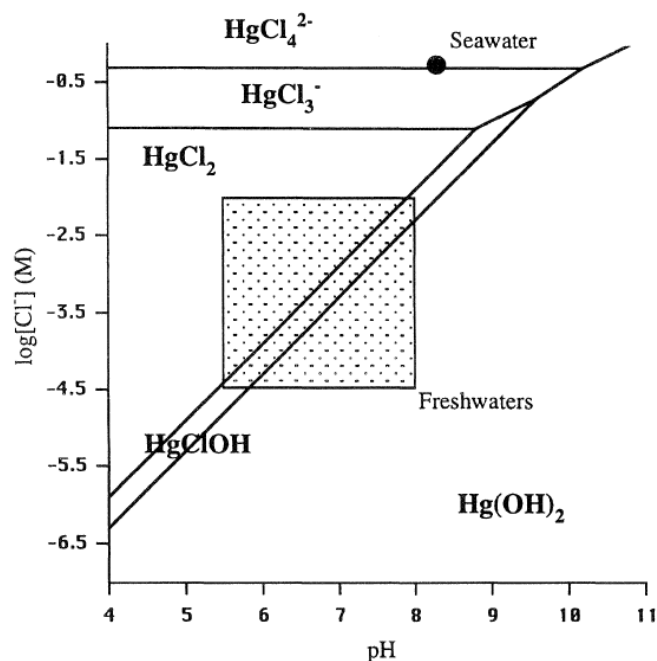


Figure 5-Speciation diagram of Hg based upon chlorine concentration and pH. Mercury chloride complexes dominate at low pH (Diagram by Morel et al., 1998).



Figure 6-CH₃Hg distillation setup showing distillation vessels on hot plates and receiver vessels in ice baths. Distillation vessels were bubbled with ultra, high-pure nitrogen.

The resulting distillate solution was aliquotted into a reaction bubbler with Millipore water and buffered to ca. 4.7 pH using sodium acetate buffer. Next, sodium tetraethylborate (NaTEB) was spiked, in

excess, into the reaction bubbler. NaTEB reacts with any CH₃Hg and Hg(II) in the sample by adding an ethyl group (-CH₂CH₃) to either Hg species to form CH₃Hg(CH₂CH₃) and Hg(CH₂CH₃). Both of these complexes are highly volatile. The volatile complexes are purged under argon onto Tenax[®] traps. The traps were burned onto a packed gas chromatography column where the methylethylmercury CH₃Hg(CH₂CH₃) was separated from other ethylated forms of Hg (Hg⁰ and Hg (II)). As the CH₃Hg(CH₂CH₃) elutes from the GC column it is decomposed to Hg⁰ via a pyrolytic trap (ca. 700 °C). The produced Hg⁰ flows into the Tekran detector where its fluorescence is measured. CH₃Hg peak areas were measured and integrated using Peak388 integration software. The peak areas were then used to calculate the final CH₃Hg concentrations.

Incorporation percentages were calculated using equation 2.1. The total concentration in the collected marine snow was divided by the sum of the total concentration in marine snow by the total concentration in the water after the marine snow was removed. The final ratio value was multiplied by 100 to convert the ratio to a percentage.

$$\frac{[Hg]_{agg}}{([Hg]_{agg} + [Water\ Hg]_{Tf})} * 100 \quad (2.1)$$

Partition coefficients (log K_d) were calculated for the marine snow data based on equation 2.2. The concentration of HgT (ng L⁻¹) in the aggregates was divided by the concentration of HgT (ng L⁻¹) in the water multiplied by the mass of the dried marine snow (kg). The log₁₀ was taken for the final ratio to determine a log K_d value.

$$\log K_D = \log_{10} \left(\left[\frac{(HgT_{agg})}{(HgT_{water} * mass)} \right] * 1000 \right) \quad (2.2)$$

Bioaccumulation of CH₃Hg and Hg(II) in mussel tissues from the uptake of Hg-contaminated marine snow

Feeding assays were conducted utilizing similar methods published by Ward and Kach (2009). Blue mussels, average 5-cm in length, were collected from Eastern Point Beach, Groton, CT (41° 19' 9.102" N, 72° 4' 5.3328" W). Any biofoulants present on the mussel shells were removed before placing them in an environmental chamber for acclimation to 18 °C over several days. Prior to the feeding assay, Velcro[®] was glued to the mussel shells using marine epoxy. Corresponding Velcro[®] pieces were glued to craft sticks so that the mussels could be attached to a craft stick in order to suspend them in the 1L jars. Mussels were chosen for the feeding assay depending upon their feeding activity prior to the assay. Their feeding activity was determined by placing them in 0.2-µm filtered seawater spiked with *Tetraselmis chui* (PLY 429 strain). The mussels were left to feed for at least 30 minutes and were chosen for the feeding assay based upon the appearance of gaping and feces in the filtered seawater indicating active feeding.

After the rolling period, jars were placed on programmable stir plates set to spin every 15 minutes for 10 seconds at 75% power. A 9" Pasteur pipet and stir bar were added to provide aeration and mixing to keep the marine snow suspended in the water column so that mussels were likely to ingest the particulates (Figure 7). Individual mussels were placed into separate jars. Once a mussel was added to a jar, 10-µm silica beads were added to serve as an indicator of clearance rate (4×10^6 beads jar⁻¹). Si beads were used in this experiment rather than polystyrene beads used in the literature (Ward and Kach, 2009; Kach and Ward, 2008; Milke and Ward, 2003) because polystyrene beads were likely to melt during the distillation of water samples for CH₃Hg analysis. The accurate collection of beads from the water samples was necessary for clearance rate calculations. Immediately following the addition of the mussels, water samples (15 mL) were collected for CH₃Hg, HgT, and bead count analysis. The mussels were allowed to feed for 2 hrs to ensure that the majority of the water was filtered. Water samples were collected again at

the conclusion of the feeding period. These water samples were used to quantify clearance rates and any changes in Hg concentration over the course of the experimental period.



Figure 7-Mussels secured on craft sticks in 1-L jars of sieved seawater spiked with CH_3Hg or HgT on stir plates used to keep marine snow in suspension.

Following the feeding trial, the mussels were removed from each jar and dissected (Figure 8). Care was taken to use a new scalpel blade for each animal. All tissues were briefly rinsed with Millipore water and dabbed dry before being stored in clean Falcon tubes. The gill, mantle, digestive gland and visceral mass, and foot and adductor muscles were collected and weighed. The wet tissues were homogenized using a Biospec titanium tissue-tearor and frozen at -20°C . Once frozen, the tissues were lyophilized for 72 hrs and stored in a freezer. Tissues were measured on the direct mercury analyzer (DMA-80) for HgT analysis before being acid digested in 4 M HNO_3 (Fisher Trace-Metal Grade) for CH_3Hg analysis.

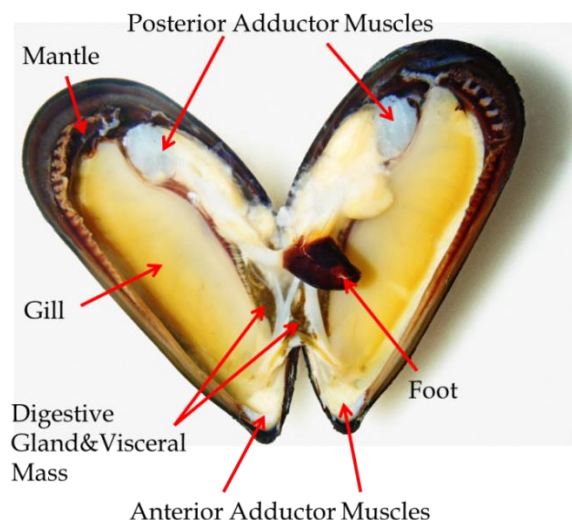


Figure 8-A dissected mussel. Labeled tissues were analyzed separately (except for the foot and adductor muscles).

Feces produced by each mussel during the feeding trial were collected, in Falcon tubes, from the jar after the corresponding animal was removed. The fecal pellets were filtered onto 0.2- μm polycarbonate filters, frozen, lyophilized, and weighed. Since the sample volume was limited, the filters were acid digested in 4 M HNO_3 such that the digest could be sub-sampled for CH_3Hg and HgT analysis.

In this experiment, the pumping rate (PR) was calculated using bead concentrations measured from the digestive gland and feces digests. The presence of beads in the digested feces and digested digestive gland indicated ingestion of marine snow and filtration of water by the mussels over the 2-hr feeding period. Beads were counted using a hemacytometer; replicate counts were accepted within a 10% occurrence. The overall PR was calculated using equation 2.3 from Kach and Ward (2008). Measuring the beads from the digestive gland and feces at the end of the experiment resulted in an underestimation of the pumping rate. The pumping rate (also referred to as the clearance rate) exponentially decreases with time, therefore samples needed to be taken at frequent times points to determine a more accurate clearance rate.

$$PR = \frac{10\mu\text{m beads ingested (No.)} / \text{initial concentration (No.ml}^{-1}\text{)}}{\text{time(hr)}} \quad (2.3)$$

The total bead count from the slide was back-calculated to the original volume of the digest for both the feces and digestive glands. The total number of beads in the feces was added to the total beads in the digestive gland to yield the total number of beads ingested. This ingestion value was divided by the original bead concentration at the beginning of the feeding experiment. The resulting ratio was divided by the time to give a final pumping rate of volume per time (equation 2.3). The measured pumping rate was then corrected to a standard mussel weight to account for physiological responses that are weight dependent (i.e., pumping rate). The standardized clearance rate was calculated using equation 2.4 from Bacon et al., (1998), where Y_s is the clearance rate for a mussel of standard weight, W_s is the standard weight of the mussel, W_e is the observed weight of the mussel, b is the weight exponent (0.70 as reported by Bayne and Thompson, 1974), and Y_e is the measured clearance rate. A standard weight of 0.5 g was used as the calculated average mussel mass in Chapter 2 and 3 was ca. 0.5 g. The calculated standardized pumping rates were used to normalize the amounts of Hg in the digestive glands and feces to the amount of volume pumped over the 2-hr feeding period. This normalization corrected the Hg concentration in the digestive gland and feces to the amount of water filtered by the mussel; therefore, correcting for any mussels that filtered much more or much less volume than other mussels.

$$Y_s = \left(\frac{W_s}{W_e} \right)^b * Y_e \quad (2.4)$$

All statistical analyses in this chapter were completed using SigmaStat 3.5 software. Two-way repeated measures ANOVA (ANOVAR) was used to compare the tissue concentrations (nmol g⁻¹ dw, not normalized to the pumping rate) due to the comparison of multiple tissues for each mussel. Two-way ANOVA analysis was used to test for possible interactions and differences between treatments (i.e., rolled and unrolled) and forms of Hg (i.e., CH₃Hg and HgT) in both the digestive glands and feces. Both ANOVAR and ANOVA analyses were followed by a full post-hoc Tukey test for multiple comparisons. All statistical tests were run using an α value of 0.05.

Results and Discussion

Statistical results and supplemental data can be found in Appendix II. Figure 25 in Appendix II shows the pumping rates of each individual animal. Table II.1 is a summary of the two-way ANOVA results. Any p values <0.05 in the ANOVA table indicate a significant difference or interaction within the data.

Incorporation of CH₃Hg and HgT into marine snow

Little to no CH₃Hg was incorporated into collected particulates from the unrolled treatments, as was expected. Maximum incorporation of CH₃Hg into marine snow was ca. 2.5% (see below; Figure 9A). CH₃Hg incorporation was higher in the rolled treatments than the unrolled. It took six days to incorporate the maximum of 2.5% of the CH₃Hg spiked in to the seawater.

Similar to the CH₃Hg experiment, almost no HgT was incorporated into the particulates collected in the unrolled treatments. Maximum incorporation of HgT into marine snow was ca. 20% (see below; Figure 9B). Incorporation was higher in the rolled treatment than the unrolled treatment, similar to the CH₃Hg experiment. It took nine days to incorporate the maximum of 20% of the HgT spiked into the seawater into the marine snow.

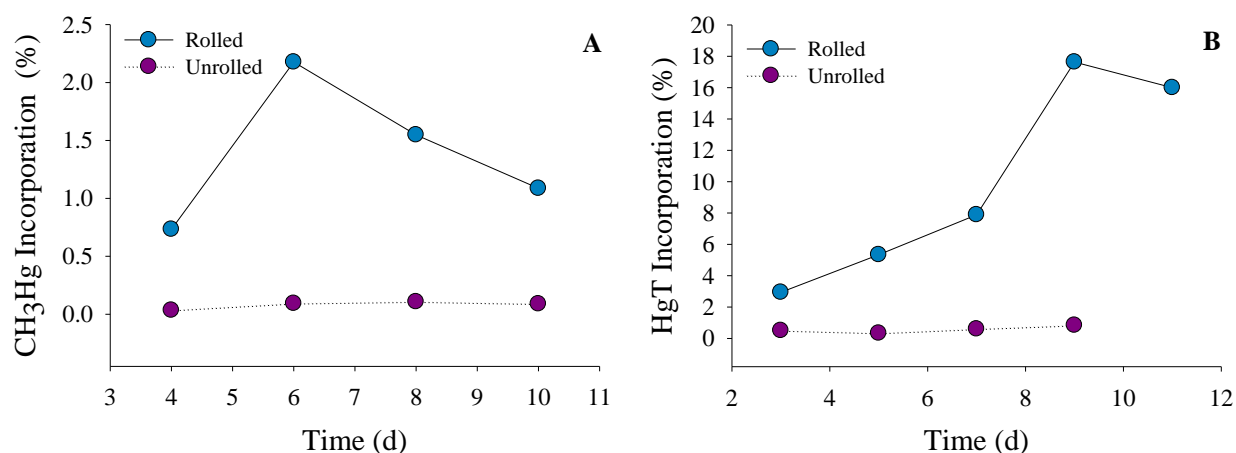


Figure 9- Incorporation of CH₃Hg (A) and HgT (B) in laboratory produced marine snow over 10 days. Data represent the mean; n=2.

The results demonstrate that CH₃Hg and HgT were successfully incorporated into marine snow in the rolled treatments and little to no CH₃Hg or HgT were incorporated in the unrolled treatments. Incorporation in both experiments was much lower than expected. It may be that low incorporation was likely due to the limited mass of aggregates produced and collected. The incorporation values calculated are based only what was collected. Therefore, these values do not account for particles remaining in the water that served as potential binding sites for the remaining Hg(II) and CH₃Hg in solution. There was a higher incorporation of HgT into marine snow than CH₃Hg in the rolled treatments. Although CH₃Hg has a strong binding affinity for thiols and organic matter it is not as particle reactive as Hg(II) (Gagnon and Fisher, 1997). Knowing that the Hg(II) is highly particle reactive, I anticipated more incorporation of HgT into marine snow than CH₃Hg (Kim et al., 2008; Lawrence and Mason, 2001). It is likely that the incorporation of CH₃Hg was limited by the amount of organic matter and TEP in the seawater at the experimental time. Both high particulate load (high TSS) and organic composition (dissolved organic matter, TEP, etc) are important to the cohesion and coagulation of particulates. The chemical composition is also important to the amount of CH₃Hg and HgT that will bind to suspended particulates. Higher incorporation of HgT may indicate the presence of higher concentrations of sulfur compounds and more organic matter.

Although more HgT was incorporated into marine snow than CH₃Hg, the calculated incorporation percentages were lower than expected based upon the known binding affinity of HgT for organic particulates. It is important to note that since the water was only passed through a 210 µm mesh, there are still various phytoplankton, microzooplankton (20 µm to 200 µm) (Calbet, 2008) and suspended materials remaining in the water. Given the experimental design, these suspended materials were not collected. These organisms and particles can bind CH₃Hg and HgT thereby decreasing the incorporation into the marine snow fraction.

Marine snow was collected after a decided settling period; therefore, the size of the particle collected was dependent on the settling velocity of particulates. Based on Stoke's Law, particles of different sizes settle at different velocities. It is also important to note that only visible marine snow was

collected from the bottom of the jars. Since I let the water settle for 15 minutes after being removed from the rolling table (rolled jars) or inverted (unrolled jars) only particulates $>300\text{ }\mu\text{m}$ were collected. As particle size increases so does the settling velocity. It was estimated that marine snow with a diameter of ca. $300\text{ }\mu\text{m}$ had a settling velocity of 0.2 mm s^{-1} (Hill et al., 2000; Hill, 1998). The 1-L jars used for this experiment were 18-cm tall. Therefore, it would take 15 minutes for the $300\text{-}\mu\text{m}$ marine snow to settle to the bottom of the jar. Smaller particles have a lower Reynold's number, meaning they are so small that they experience more friction with water (i.e., drag) such that they settle slower because the water is more viscous. In order to collect smaller particulates, $<300\text{ }\mu\text{m}$ in size, the settling time would need to be increased, or the smaller particulates would need to be collected by filtration. Therefore, the sampling process used in this experimental design does not account for the suspended particulates remaining in the water once the visible, marine snow has been collected. This may skew the incorporation data to appear as though the incorporation was much less than what was truly incorporated in to all of the particulates available in the water.

The theory that incorporation was low due to most of the CH_3Hg and Hg(II) remaining bound to particulates in suspension was tested by evaluating the partition coefficients ($\log K_d$) of CH_3Hg and Hg(II) in relation to the marine snow. The partition coefficients, $\log K_d$, were calculated for both rolled and unrolled treatments in the HgT experiment and compared to published partition coefficients (Fitzgerald et al., 2007; Coquery et al., 1997; Stordal et al., 1996). The resulting $\log K_d$ values (ca. 10^5 L kg^{-1} for each) were similar to the published values albeit on the lower end of the reported range. A higher $\log K_d$ value indicates a higher partitioning onto particulates than in to the dissolved phase. The similar and high $\log K_d$ values for both rolled and unrolled treatments may indicate the presence of particulates in the water that, despite whether or not marine snow was produced, the HgT was bound.

Dissolved organic carbon (DOC) concentrations may have an additional effect on the binding capacity when comparing CH_3Hg and HgT . The CH_3Hg incorporation experiment was conducted in May with a DOC concentration of $5.0\text{ }\mu\text{M C}$. The HgT experiment was conducted in January with a DOC concentration of $10.4\text{ }\mu\text{M C}$. These concentrations are much lower than expected. This may be due to a

long sample holding time or incomplete conversion of organic carbon to CO₂ when analyzed.

Nonetheless, I could detect a difference in DOC between experiments and compare the values. The DOC for the HgT experiment was twice as high as the CH₃Hg experiment. The lower organic carbon concentration may have limited the available binding sites for CH₃Hg resulting in a lower incorporation compared to HgT incorporation.

Bioaccumulation of CH₃Hg and Hg(II) in mussel tissues from the uptake of Hg-contaminated marine snow

Bioaccumulation was quantified by determining the concentrations in the various tissues and feces. The concentrations of CH₃Hg and HgT were compared amongst the collected tissues: gill (G), mantle (M), digestive gland and visceral mass (DG), and foot and adductor muscles (F&A) (see below; Figures 10A and 10B). Repeated measures two-way ANOVA (ANOVAR) analysis of the CH₃Hg tissue data (Figure 10A) indicated a significant interaction between the treatment (rolled and unrolled) and the tissue (G, M, DG, FA) ($p < 0.05$). This implies that the concentration in the tissues was dependent on the treatment the mussel was exposed to (i.e., rolled or unrolled), but there was no significant difference ($p > 0.05$) between the tissues from rolled or unrolled treatments. Within the rolled treatments, there was a significant difference between the DG, and the G, and the FA tissues ($p < 0.05$), but there was no significant difference between the DG and M, nor between the M and the G and the FA. Significances are indicated in both Figures 10A and 10B by the letters above their respective bars. Tissues with a significant difference have different letters; those that are not significantly different have the same letter. ANOVAR analysis of HgT tissue concentrations indicated no significant interactions ($p > 0.05$) (see below; Figure 10B). There was no significant difference between rolled and unrolled treatments within the tissues, but there was a significant difference between the DG and the rest of the tissues ($p < 0.05$). There was also a significant difference between the FA and the M in both treatments ($p < 0.05$). Since there was no interaction between the treatments and the tissue the lines drawn above the bars are used to group the

treatments together. The letters above the lines indicate significant differences between the tissue groups; those that have no significant difference have the same letter.

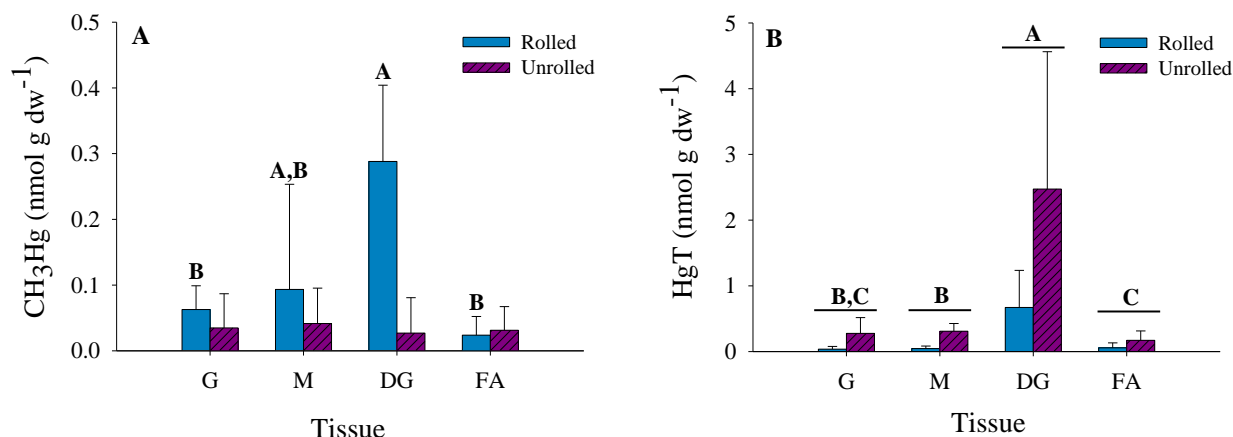


Figure 10- CH₃Hg (A) and HgT (B) tissue data. Two-way ANOVAR analysis of CH₃Hg tissue concentration indicated a significant interaction ($p < 0.05$) between the treatments and tissues. A full post-hoc Tukey test indicated no significant difference within the treatments ($p > 0.05$), but did indicate a significant difference between the tissues within the rolled treatment ($p < 0.05$). Two-way ANOVAR analysis of HgT tissue concentration indicated no significant interaction between the treatments and tissues ($p > 0.05$) nor any significant difference between the treatments ($p > 0.05$). There was a significant difference between the tissues overall indicated by the lines drawn above the bars. Letters above the drawn lines indicate significant differences between tissues. Those that share the same letter are not significantly different; those with different letters are significantly different. The data represent the mean \pm SD, $n=4$.

The tissue data for both the CH₃Hg and HgT treatments (Figures 10A and 10B) indicate no significant difference between the rolled and unrolled treatments. This lack of difference between treatments can be attributed to several factors. Variability in the background animal tissue concentrations caused analytical difficulties with overcoming the signal to noise ratio (S:N). Therefore, the lack of significant difference between the rolled and unrolled treatments may be limited to the analytical variability and the relatively small spike concentrations used in this experiment. Second, the variability of the marine snow produced in each jar, both the size and the composition, may have affected the capture efficiency and physical uptake of CH₃Hg and HgT from the marine snow. If the incorporation of CH₃Hg and HgT caused the marine snow to become too compact for the mussel to break up then it is possible

they were unable to ingest the marine snow in the rolled treatments. It is possible that due to the strong binding relationship between CH_3Hg and Hg(II) and organic matter could have made the marine snow more compact as CH_3Hg and Hg(II) were incorporated. Another possible theory accounting for compact marine snow is that the rolling process caused the marine snow to tightly coagulate. The small jars and rolling speed could have created tighter, more compact marine snow than occurs in the environment. Third, recall that mussels have a capture efficiency of ca. 100% for particles $\geq 6 \mu\text{m}$ in size (Ward and Shumway, 2004). As the water was passed through a 210- μm sieve, it is likely that there were particles and plankton $\geq 6 \mu\text{m}$ in the unrolled treatments that had CH_3Hg and HgT bound to them or biomagnified in their cytoplasm or tissues. If there was a high concentration of these bioavailable particles or organisms in the water the mussels would have been able to capture and ingest these particles even in the absence of marine snow.

In both Figures 10A and 10B, the DG demonstrated a significantly different concentration of either form of Hg from the other tissues. This result indicates the successful ingestion of contaminated marine snow (rolled treatment) and particulates (unrolled treatment), showing that the mussels did not reject the marine snow fed to them. In Figure 10A the CH_3Hg DG concentration was significantly greater in the rolled treatment than in the unrolled treatment and significantly greater than the G and the FA. This indicates that the mussels ingested more CH_3Hg from the marine snow in the rolled treatment than from the particulates in the unrolled treatment. But the remaining tissue concentrations were not significantly different between treatments as was expected because of the acute exposure. In Figure 10B, the HgT DG concentrations were greater than the other tissues, but there was no difference between the treatments. Again this data indicates that there may have been enough particulates bound with HgT in the unrolled treatment for the mussels to capture and ingest, thereby eliminating the difference between the presence and absence of marine snow. Statistical analyses indicated that if more mussels were exposed to the marine snow this would possibly strengthen the statistical evidence of the positive trends; therefore more mussels were utilized in the following isotope experiment.

The concentrations in the DG and feces needed to be normalized to the pumping rate to correct for any mussels that filtered more water than others. Pumping rates were calculated from bead counts measured from DG and feces digests using equation 2.3. Although other mussel feeding studies calculate clearance rates from changes in bead concentrations in the water (Coughlan, 1969), I chose to use the method by Kach and Ward (2008) because the Si beads had a high settling rate (equivalent to 500 ml hr⁻¹) which negatively skewed the filtration calculations. The calculated filtration rates ranged from 0.3-7.3 L hr⁻¹ g⁻¹ with an average of 2.2 ± 1.8 L hr⁻¹ g⁻¹. The pumping rates were standardized to a standard mussel weight to correct for weight dependent physiological responses (equation 2.4). CH₃Hg and HgT tissue (Figure 11) data and feces data (Figure 12) were normalized to the standardized pumping rates.

The total concentrations from each tissue were added together to yield a total body concentration. The total body burden was normalized to the standardized filtration rate (equation 2.4) for the entire 2-hr feeding period. Figure 11 (see below) shows the normalized concentration of the entire mussel based on what volume of water they filtered assuming that the volume filtered indicates the amount of marine snow ingested. Two-way ANOVA analysis indicated a significant interaction ($p < 0.05$) between the treatment and the type of Hg. There was no significant difference ($p > 0.05$) between CH₃Hg and HgT in the rolled treatment, but there was a significant difference between CH₃Hg and HgT in the unrolled treatment. There was also a significant difference between CH₃Hg in the rolled and unrolled treatment and between HgT in the rolled and unrolled treatment ($p < 0.05$). Significant differences are indicated in Figure 11 by different letters in above the bars. Bars with similar letters are not significantly different.

On a per gram basis the HgT concentrations in the DG were much greater than the CH₃Hg (Figure 10), but once corrected to the volume each mussel pumped and for mass-dependent physiological response, the values are within the same order of magnitude. These results imply that the HgT mussels filtered more water than the mussels exposed to CH₃Hg. This does not mean that the mussels fed more because there was HgT in the water, but rather, this indicates that mussels need to filter more water in order to absorb as much HgT as CH₃Hg into their tissues. These results would agree with the literature

that has demonstrated organisms depurate more Hg(II) than CH₃Hg and therefore exposure to more Hg(II) is required to induce greater bioaccumulation (Sauvé et al., 2002; Clarkson and Magos, 2006).

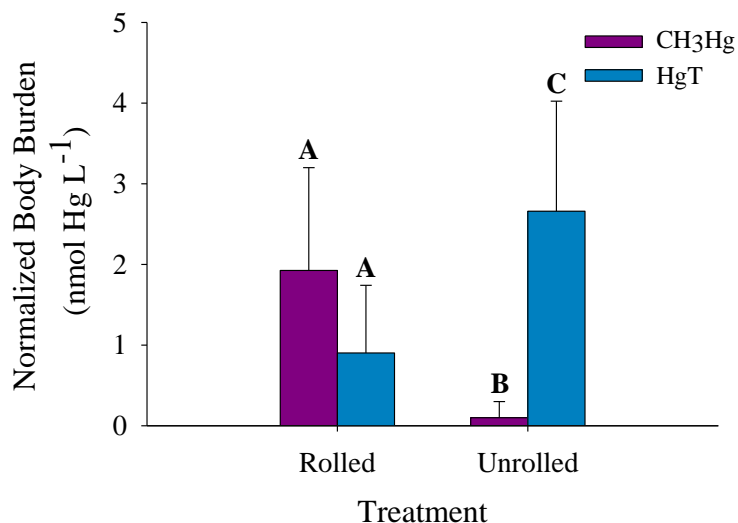


Figure 11- Average of total soft tissue CH₃Hg and HgT concentrations normalized to the standardized filtration rates (nmol Hg L⁻¹ during the 2-hr feeding period). Two-way ANOVA analysis indicated a significant interaction between treatment and spike ($p < 0.05$). There was no significant difference between CH₃Hg and HgT in the rolled treatment ($p > 0.05$), but there was a significant difference between CH₃Hg and HgT in the unrolled treatment ($p < 0.05$). There was a significant difference between the rolled and unrolled treatments for CH₃Hg and HgT. Data represents the mean \pm SD; $n=4$.

The normalized body burden data in Figure 11 indicate a higher CH₃Hg body burden in the mussels exposed to the marine snow in the rolled treatments, but a higher HgT body burden in the mussels exposed to the unrolled treatments. These data agree with the digestive-gland data presented in Figures 10A and 10B where the concentration in the DG was significantly greater than the other collected tissues. The elevated CH₃Hg body burden in the rolled treatment indicates that the mussels were ingesting more CH₃Hg from marine snow than from the particulates in the unrolled treatments, whereas the mussels exposed to HgT absorbed significantly more from the particulate in the unrolled treatments. This result rejects my hypothesis that marine snow serves as a pathway of HgT uptake into mussels, except that these tissue concentrations only reflect what occurred within the 2-hr feeding period. Therefore, these

normalized concentrations do not indicate whether or not the mussels truly assimilated the contaminated marine snow.

Assimilation efficiencies of CH_3Hg or Hg(II) into the mussels could not be calculated from the collected data because there was no depuration period conducted. Bivalves produce two types of feces, intestinal and glandular feces. Intestinal feces are produced within 30 minutes to 2 hrs of feeding, but glandular feces undergo a longer metabolic process. Mussels select for particles for ingestion on their labial palps (Ward and Shumway, 2004, Ward, 1996). If selected for, the particles are transported to the mouth for ingestion. Once in the stomach, the particles undergo extracellular digestion. First, the particles are ground between the crystalline style and the gastric shield. As the crystalline style grinds into the gastric shield, digestive enzymes are released to break down particulates into very fine sizes. These fine particles are then sorted by cilia in the stomach. Finer particles remain in suspension as the cilia beat while heavy, dense particles settle into fine ridges and grooves and are transported into the intestine for rejection (intestinal feces). The particles that remain in suspension are swept to the digestive gland duct openings. Once the particles are brought into the digestive gland they undergo intracellular digestion. Digestive cells envelope particles and are digested in vesicles called lysosomes. These enzyme filled cells digest the particles. The products of this digestion are released into the haemolymph system. Eventually the digestive cells burst releasing waste products that are transported to the intestine for depuration (glandular feces) (Ward and Shumway, 2004; Owen, 1966). Since we only collected feces immediately after the feeding assay it is likely I collected either intestinal feces or feces containing particles from before the experiment. In summary, the feces data may not be an accurate portrayal of ingestion of CH_3Hg and Hg(II) contaminated marine snow by the mussels. In order to get an accurate account of assimilation efficiency we will need to collect feces at 36 hr and 72 hr after the mussels are exposed to the contaminated marine snow in order to account observe complete metabolic processing of particulates.

Fecal pellets, collected after the feeding period, were analyzed for both CH_3Hg and HgT (Figure 12). The feces concentrations were normalized to the calculated pumping rate similar to the normalized body burdens. These values reflect the amount of CH_3Hg and HgT depurated during the

feeding period depending on the volume of water filtered during the feeding period and, potentially, the amount of marine snow ingested. Two-way ANOVA analysis of CH₃Hg and HgT indicated no significant interaction; therefore, the concentration in the feces was not dependent upon the treatment. There was no significant difference between rolled and unrolled treatments ($p>0.05$) for each form of Hg, but there was a significant difference between the CH₃Hg and HgT concentrations within each treatment ($p<0.05$). Significant differences are indicated by different letters above the bars in the figure. Bars with the same letter are not significantly different.

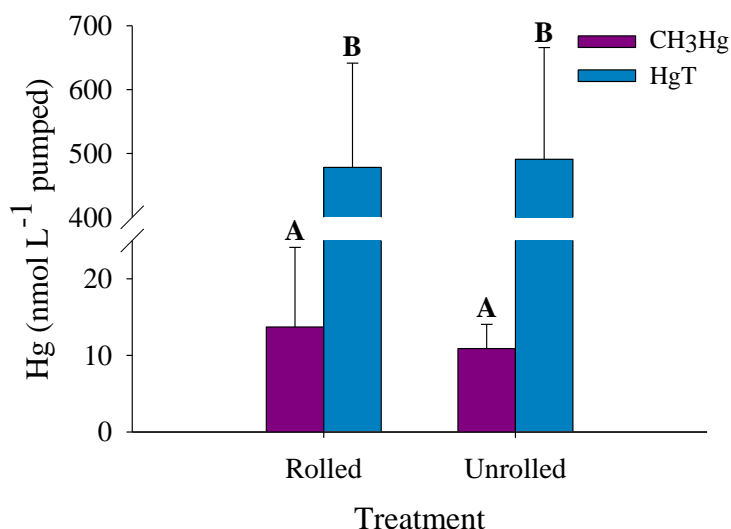


Figure 12- Feces concentrations corrected for weight dependent physiological response (equation 2.4). The concentrations compared reflect depuration of CH₃Hg and HgT during the feeding period. Letters above the bars indicate significance; different letters indicate significant difference ($p<0.05$), whereas similar letters indicate no significant difference ($p>0.05$). Data represent the mean \pm SD, $n=4$.

The feces data presented in Figure 12 demonstrate no significant difference between the presence and absence of marine snow (i.e., rolled and unrolled), but there is a strong significance between the concentrations of the Hg forms present in the feces. The CH₃Hg and HgT concentrations in the feces can be used as a proxy of CH₃Hg and HgT ingested by the mussels. Higher concentrations of HgT than CH₃Hg were observed in the feces indicating a higher rate of depuration of the inorganic mercury compared to the organic mercury. Data from the literature state that Hg(II) is depurated at a much faster

rate than CH₃Hg due to the different bioavailability of either form. Hg(II) is less bioavailable than CH₃Hg because it forms strong bonds with particulates which are difficult to break (as indicated by its high K_d). Whereas, the bonds formed between CH₃Hg and particulates are not as strong. Bioavailability is correlated with environmental pH; therefore any changes in pH in the mussels' digestive glands may affect the partitioning, assimilation, and depuration of different forms of Hg (Griscom et al., 2002; Gagnon and Fisher, 1997). McAloon and Mason (2003) and Lawrence et al. (1993) concluded that CH₃Hg was more readily solubilized than Hg(II) in the gut of sea cucumbers and subsequently more accumulation of CH₃Hg occurred. Griscom et al. (2002) reported gut pH values of ca. 5.6 in *Mytilus edulis* and higher metal release in the gut juices than in a control seawater exposure (pH 5.5); therefore the acidity of the gut released more of the metals such that they could be readily accumulated in the animal tissues. Since there was a higher depuration of HgT compared to CH₃Hg my results concur with the published trends presented.

Conclusions

Incorporation of CH₃Hg and HgT into marine snow

In conclusion, both CH₃Hg and HgT were incorporated into laboratory produced marine snow; therefore, my hypothesis was confirmed. The results of this study demonstrate that it is probable that CH₃Hg and HgT are readily incorporated into marine snow present in the open environment. To account for the uptake of CH₃Hg and HgT in biota or larger particulates, future experimentation may require a finer initial sieving (<210-μm mesh). Care must be taken in sieving the water through too fine of a mesh. If too much inorganic and organic material is removed from solution marine snow will not form. Alternatively, more particulate fractions could be collected after the removal of the marine snow and analyzed (i.e., ≥10 μm particulates, 0.2- to 10-μm particulates, and the dissolved phase <0.2 μm). By analyzing all of the particulate fractions it may be possible to determine a proper mass balance for particulate-bound CH₃Hg and HgT and what fraction is still bioavailable after the production of marine

snow. The high variability experienced in this experiment may also be corrected for if stable Hg isotopes are used in future experiments. Stable Hg isotopes are widely used as tracers to detect small changes at environmentally relevant concentrations.

Bioaccumulation of CH₃Hg and Hg(II) in mussel tissues from the uptake of Hg-contaminated marine snows

In conclusion, I can state that my hypothesis that marine snow can serve as a pathway for Hg uptake in blue mussels has been tentatively confirmed. Figures 10A, 10B, and 11 show the uptake of CH₃Hg and HgT from both the marine snow in the rolled treatments and particulates in the unrolled treatments. More CH₃Hg was absorbed from the marine snow than from the particles in the unrolled treatments while the opposite was observed for HgT. There was only a significant difference between the uptake of CH₃Hg from the rolled and unrolled treatments, whereas the uptake of HgT did not depend on the treatments. This result implies that CH₃Hg is more bioavailable once bound to marine snow, but HgT can be absorbed when bound to any particulate that the mussels can capture. I hypothesize that the lack of significant difference between the rolled and unrolled treatments for HgT is due to the presence of bioavailable particulates (>6 µm) remaining in the water after marine snow is produced.

The feces data presented in Figure 12 indicate depuration of CH₃Hg and HgT during the feeding assay. Although official assimilation efficiencies could not be collected due to the experimental design, the feces data show high initial depuration over the course of the feeding experiment. As was expected, the initial depuration of HgT was greater than for CH₃Hg since CH₃Hg is readily bioaccumulated through the foodweb (Lawrence and Mason, 2001). Although it is difficult to elucidate clear trends from the data due to low sample numbers and high background concentrations, I can still use the feces data as a proxy for ingestion of both forms of Hg.

The mussels ingested and initially absorbed CH₃Hg from marine snow and HgT from both marine snow and bioavailable particulates. This conclusion was further supported by the presence of CH₃Hg and

HgT in collected feces indicating that ingestion had occurred during the 2-hr feeding period. Further studies utilizing stable Hg isotopes were conducted to clarify the trends observed in this chapter. Utilizing stable Hg isotopes will allow me to expose more mussels to both forms of Hg simultaneously for most robust statistical analysis and to reduce variability due to differences in CH₃Hg and HgT background concentrations in the tissues.

Chapter 3-Quantifying assimilation of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT stable isotopes from marine snow in blue mussels

Introduction

In the previous experiment, the results were tempered by the lack of substantial changes in tissue concentration above the background concentration in the mussels. To address this, it was decided to use isotope spike additions as these would allow differences to be ascertained even at low exposure concentrations, rather than use higher exposure concentrations. Mercury has seven stable isotopes: ^{202}Hg , ^{200}Hg , ^{199}Hg , ^{201}Hg , ^{198}Hg , ^{204}Hg and ^{196}Hg in descending natural abundance (Kritee et al., 2007, Bergquist and Blum, 2007). Only the stable isotopes $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT were used in this experiment as tracers. $^{199}\text{CH}_3\text{Hg}$ has a natural abundance of 16.8% and ^{200}HgT has an abundance of 23.10% (Hintelmann et al, 1997). These isotopes can be measured using inductively coupled plasma mass spectrophotometry. This method is considered to be more sensitive than CVAFS used in the previous chapter because the relative enrichment of the isotope is greater than the change in total concentration. The increased sensitivity allows for the use of lower (more environmentally relevant) spike concentrations in feeding experiments while still yielding a detectable signal above the background concentrations (Hintelmann and Evans, 1997). In addition to decreasing the concentrations of Hg mussels were exposed to, I was able to expose mussels to both CH_3Hg and HgT within one jar rather than in separate jars further eliminating experimental variability. Simultaneous exposure to both forms of Hg increased the number of mussels that could be fed contaminated marine snow within one experiment. The simultaneous exposure could also indicate any changes in isotope ratio within the marine snow and mussel tissues which could indicate differences in the assimilation of Hg(II) relative to CH_3Hg .

Materials and Methods

Experiment Preparation and Execution

All materials were trace-metal cleaned before conducting the experiments. Materials included: 1-L glass rolling jars, 0.2- μm and 8- μm polycarbonate filters, 15-mL and 50-mL polyethylene centrifuge tubes, 25-mm and 47-mm glass frit filters, graduated filter holders, Erlenmeyer flasks, magnetic stir bars,

9" Pasteur pipets, craft sticks, Velcro[®], Marine Tex[®], coulter cups, and 15-mL and 50-mL glass centrifuge tubes.

Marine snow was produced using the same methods described in Chapter 2, but with the following modifications to the method. The seawater was passed through a 100- μ m mesh sieve rather than a 210- μ m mesh used in the previous experiment to remove more of the large microzooplankton from the water to prevent the possible bioconcentration artifact I suggested in Chapter 2. The Hg concentrations were changed to 0.25 nM $^{199}\text{CH}_3\text{Hg}$ (synthesized from a $^{199}\text{HgCl}_2$ standard reacted with cobalamine) and 0.25 nM ^{200}HgT (prepared from a HgCl_2 solution). Rather than running one experiment two groups of 24-mussels were fed on two separate days for 2-hr feeding periods because of various logistical issues. I anticipated being able to pool the data together from both days, but natural variability was too great, as discussed further below. Finally, the rolling period was decreased from eight days to four days for this experiment. I hypothesized that leaving the seawater in the jars for an extended period could have created an ideal environment for anaerobic methylation which would create an artifact in the data.

Experimental controls consisted of three different treatments: a bead settling control, two marine snow controls, and a Hg control. The bead settling controls consisted of three jars, filled with 100- μ m sieved seawater, spiked with 10- μ m polystyrene beads (10×10^6 beads jar⁻¹). Polystyrene beads were used in this experiment rather than silica beads because, as mentioned in Chapter 2, the silica beads had a higher density and rapidly settled out of the water over the feeding period. Duplicate water samples were collected at the beginning of the feeding period and at the conclusion of the 2-hr feeding period. One replicate of each water sample was directly counted on the coulter counter. The other replicate of each water sample was filtered through an 8 μ m polycarbonate filter and re-suspended with 0.2- μ m filtered seawater into acid clean coulter cups before being counted. The filtrates were saved for Hg analysis. The marine snow controls accounted for marine snow production and Hg incorporation. One set of controls accounted for the actual production of marine snow (i.e., rolled jars versus unrolled, stationary jars). The unrolled jars had no visible marine snow on the bottom of the jars. The other control consisted of six jars

filled with the 100- μm sieved seawater. Three jars were rolled and three jars remained on a stationary surface (unrolled). All of the jars were spiked with 0.25 nM $^{199}\text{CH}_3\text{Hg}$ and 0.25 nM ^{200}HgT ; but no mussels were added. These were used to determine how much Hg was incorporated into the marine snow at the time of the feeding assay. The Hg control consisted of six unrolled jars without any Hg spiked into them and a mussel was added to each. Data from the previous experiment indicated that there was no significant difference between rolled and unrolled treatments for controls without Hg; therefore we did not roll any Hg control jars.

The jars and mussels were kept at 18 °C in an environmental chamber under a light/dark cycle. At the end of the rolling period, rolled jars were removed from rolling tables and placed onto stir plates. Both rolled and unrolled jars were gently inverted three times and then opened. Marine snow controls, that mussels were not added to, were placed on stir plates and allowed to stir similar to the other jars. For the remaining exposures the jars were opened and then pipets, stir bars, mussels, and 10- μm polystyrene beads (6×10^6 beads jar $^{-1}$) were added. After the mussels and beads were added, the stir plates were turned on and duplicate water samples were immediately collected. The mussels were secured onto craft sticks and added to the jars, similar to Chapter 2, (Figure 7) and allowed to feed for 2 hrs. At the conclusion of the 2-hr feeding period duplicate water samples were collected for Hg analysis and clearance rate analysis.

After the 2-hr period, the marine snow control jars were removed from the stir plate and allowed to settle for 15 minutes. After settling, the visible marine snow (in rolled jars) was collected from the bottom of the jar using a Pasteur pipet. The settled particulates in the unrolled jars were randomly collected from the bottom of the jar similar to the method described in Chapter 2. After T_f for the mussel exposures, mussels were removed from their respective jars and placed into clean tri-corner beakers filled with 0.2- μm filtered seawater. Each mussel was fed 10^6 cells of phytoplankton culture (*Tetraselmis chui*) each day up to 72-hrs post-feeding to maintain health and to flush the CH_3Hg and HgT from the mussels' guts (Figure 13). Six control animals and six Hg-exposed animals (three rolled, three unrolled) were immediately collected and frozen following the conclusion of the feeding period to establish the T_0

concentrations in the digestive glands. Hereafter, T_0 refers to the time period at the conclusion of the feeding period. Feces present in all of the feeding jars were also collected after the mussels were removed at the conclusion of the feeding period. Mussels placed into the tri-corner beakers were removed 12, 36, and 72 hrs after the conclusion of the feeding assay. These mussels were frozen and dissected later for Hg analysis. Feces were collected from each tri-corner beaker at each time point until its respective mussel was removed. After feces were collected the water was changed with new 0.2- μ m filtered seawater. Remaining control mussels were sacrificed at 72 hrs. All collected samples (other than water) were frozen for future sample preparation. Water samples were preserved with 0.5% HCl (Fisher Trace-Metal Grade) and kept refrigerated in the dark..

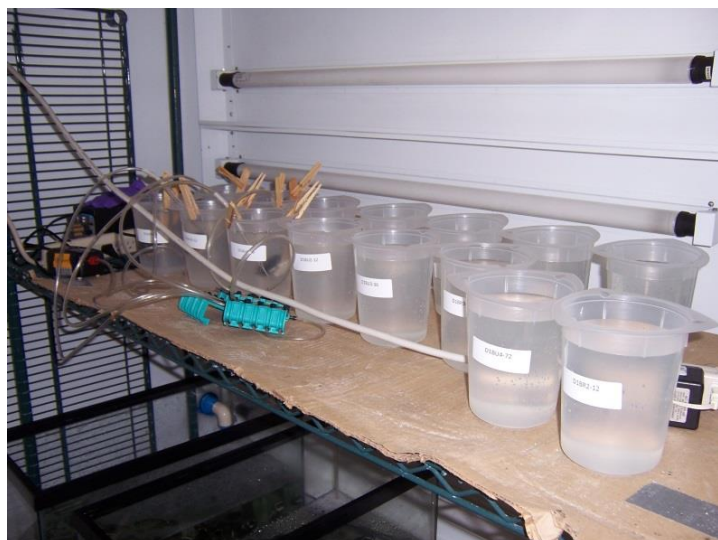


Figure 13-Plastic tri-corner beakers filled with 0.2- μ m filtered seawater. Mussels were transferred into clean beakers for 12, 36, and 72 hrs after the conclusion of the feeding period. Mussels were fed *Tetraselmis chui* daily to maintain health and to flush CH_3Hg and HgT from the mussels' guts.

Analytical Preparation

The analytical techniques in this study differ from Chapter 2 because a Perkin-Elmer DRC II Inductively Couple Plasma Mass Spectrometer (ICPMS) was used as a detector rather than the Tekran[®] CVAFS detector. The ICPMS is capable of separating the various isotopes based on their mass-to-charge ratios (m/z) by utilizing a quadrupole mass analyzer and electron multiplier transducer. The quadrupole consists of four parallel cylindrical rods that can be charged to serve as electrodes. Two of the rods are

positive while the other two are negative. The rods are programmed to maintain specific charges such that when ions, not of the desired m/z , are accelerated into the space between the rods they are deflected from the main pathway into the rods converting them into neutral species. This means that only atoms of a specific m/z actually make it through the channel between the rods and to the transducer which converts the ions into an electrical signal. Once the ions are converted into electrons in the transducer, the number of electrons is increased exponentially every time the electrons hit a consecutive cathode plate. For every single ion that enters the transducer a large number of electrons are detected by the instrument yielding an analytical signal (Skoog et al., 2007). The electrical signal detected is converted by the computer into either a chromatogram (CH_3Hg analysis) or intensity counts (HgT) which can be back-calculated to actual concentrations.

CH_3Hg analysis was identical to the CVAFS analysis other than the detector being an ICPMS rather than a fluorescent detector. HgT analysis differed from the CVAFS technique because a flow injection analysis system (FIAS) (Figure 14) was utilized instead of the bubbler technique described in Chapter 2. The FIAS system is programmed to sample 4.5-mL aliquots of sample and mix it with 3% HCl (Fisher Trace-Metal Grade) carrier matrix. The solution is then mixed with 1.5% SnCl_2 (Fisher, suitable for mercury analysis) in 3% HCl (Fisher Trace-Metal Grade) to reduce any free Hg(II) to Hg^0 . This reduced solution is purged with Ar in a small reaction vessel. Volatile Hg^0 diffuses across a gas-liquid separator filter and then is transported by Ar carrier gas into the ICPMS. Prior to FIAS analysis, all samples were spiked with hydroxylamine pre-reductase.



Figure 14-The flow injection analysis system (FIAS, indicated by solid arrow) connected to a Perkin-Elmer ELAN DRC-II ICPMS used for ^{200}HgT analysis. The dashed arrow shows the lit plasma where gaseous sample enters the ICPMS.

Quality control for the CH_3Hg analysis included running analytical duplicates every 10 samples per bubbler and analyzing digested Tort-2 standard reference material (specified in Chapter 2). Quality controls for CH_3Hg water distillation included distillation duplicates, matrix spikes, and preparatory blanks. Quality control for HgT analysis consisted of running an analytical duplicate, a 4.9-nM standard check (total dissolved HgCl_2 , non-isotope specific), a matrix blank, and Mess-3 and Tort-2 (specified in Chapter 2) SRMs were analyzed every 10 samples. In addition to the analytical quality controls, an internal ^{201}HgT standard was spiked into each sample to a final concentration of 4.9 nM. Measured values of the ^{201}HgT were used in isotope dilution calculations to determine concentrations of each HgT isotope and to monitor variability and instrument drift.

The frozen marine snow samples were lyophilized and a dry mass was measured. The dried filters were digested in 10-mL of 4.57 M HNO_3 in a 60 °C water bath overnight. A 1-mL aliquot of the original 10-mL digest was directly analyzed for CH_3Hg . Acid digests directly analyzed for CH_3Hg were neutralized with 10 M potassium hydroxide (KOH) and then adjusted to a pH of 4.9 with 4 M acetate buffer. This pH-adjusted solution was reacted with NaTEB such that any Hg^0 in the sample was ethylated for GC separation onto the ICPMS. A 2.5 mL aliquot of the 10-mL digest was oxidized with BrCl and

incubated at 60 °C overnight for HgT analysis. The oxidized digest was diluted up to 12 mL with MQ-H₂O.

The frozen mussels were gradually thawed and then carefully dissected to minimize loss of delicate tissue. Similar to Chapter 2 the gill (G), mantle (M), digestive gland and visceral mass (DG), and the foot and adductor muscles (FA) were individually collected, except the gill and mantle were grouped together in this experiment. Each tissue was rinsed with MQ-H₂O and patted dry before weighing. Each sample was homogenized using the same tissue-tearor as in Chapter 2 and the resulting tissue slurry was frozen at -20 °C and lyophilized. Dry masses of all tissues were measured. Attention was focused on the DG as this was the only tissue that showed a change in concentration in Chapter 2. Dried digestive glands were digested in 7-mL of 4.57 M HNO₃ and incubated in a 60 °C bath overnight. Just like the marine snow samples, a direct aliquot (100 µL) of digest was analyzed for CH₃Hg. A 1mL aliquot of the tissue digest was oxidized with BrCl and incubated overnight and then diluted up to 10 mL with MQ-H₂O.

Feces samples were thawed and filtered onto 0.2-µm polycarbonate filters. The feces were then rinsed with NH₄COOH to exchange salts from the feces to acquire a more accurate weight. The filters were frozen at -20°C and then lyophilized for 72 hrs. Dried filters were weighed and then acid digested overnight in 10-mL of 4.57 M HNO₃. An aliquot (100 to 400 µL) of digest was directly analyzed for CH₃Hg. Similar to the other digested marine snow and digestive glands, 2.5-mL of the 10-mL digest was oxidized with BrCl and incubated at 60 °C overnight. The oxidized solution was diluted to 12 mL with MQ-H₂O.

Water samples were distilled for CH₃Hg analysis similar to Chapter 2. Aliquots of the collected distillate (5 to 15 mL) were directly spiked into MQ-H₂O. The samples were buffered to a ca. 4.9-pH and then NaTEB was spiked into the solution as described in Chapter 2. The same trap to GC connection was used as described in Chapter 2 except the gaseous species of Hg⁰ eluted into the ICPMS rather than the CVAFS Tekran detector. Water samples for HgT analysis were directly analyzed using the FIAS system. The preserved water samples were oxidized with BrCl and allowed to incubate at room temperature overnight and then analyzed.

Data Analysis and Statistical Analysis

All isotope concentrations were calculated using isotope dilution ratios between the isotopic tracers (i.e., $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT) and ambient Hg (which was estimated based on the ^{202}HgT signal and natural abundance) in the sample as presented by Hintelmann and Evans (1997). Utilizing the raw ICPMS peak areas or intensity counts (CH_3Hg and HgT ; respectively) and the known natural abundance of each isotope I was able to calculate the amount of excess isotope based on the ambient ^{202}Hg signal. The excess isotope concentrations calculated were the concentration of spike remaining in the water, of spike incorporated into the marine snow, of spike assimilated into the digestive gland, or of spike depurated in the feces. The method detection limits (MDL) for the CH_3Hg and HgT analyses were calculated by taking 3x the RSD of the 199:202 (for CH_3Hg) or 200:202 (for HgT) ratio from the measured samples (raw signal) and multiplying it by the background concentration (average ^{202}Hg raw signal) and the natural abundance of the analyzed tracer isotope (Hintelmann and Evans, 1997). Any samples below the MDL were not considered to have a significantly greater concentration than the background (EPA 40-CFR Parts 136 Appendix B). Quality control and quality assurance results are detailed in Table **.

Incorporation of both isotopes ($^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT) into the marine snow was calculated using equation 2.1. The $\log K_d$ was calculated using equation 2.2. The resulting values are detailed in table II.1.

Recall that one replicate of all collected water samples were filtered through an 8- μm acid cleaned polycarbonate filter. The water samples were filtered because the polystyrene beads were likely to melt or deform during the CH_3Hg distillation procedure and the beads were needed to calculate the clearance rates. The filtrates were saved for Hg analysis and the filters were rinsed with 0.2- μm filtered seawater into acid cleaned coulter cups. The re-suspended bead solutions were counted using a coulter counter with a 100- μm aperture with a size window set to 5.49 to 19.40 μm and set to measure 0.5-mL aliquots of sample over 13 seconds. Each sample was counted at least in duplicate with a 10% maximum difference. The averages of the bead counts were used in equation 3.1 to determine the clearance rate for each animal. Note that a different equation was used to calculate clearance rate in this chapter than the

equation by Kach and Ward (2008) in Chapter 2. I used the equation published by Coughlan (1969) for this data because the polystyrene beads used in this experiment and did not have the settling problem as when the silica beads. In the literature, clearance rate is calculated using equation 3.1, where C_o is the bead concentration at the start of the feeding time, C_t is the bead concentration at the end of the feeding time, m is the filtering rate of a single animal, n is the number of animals, M is the volume of suspension, a is the settling rate of the particles, and t is the time of the feeding period (Coughlan, 1969). Retention for particles $>6 \mu\text{m}$ is ca. 100% in the blue mussel, therefore any beads missing from final water samples compared to initial bead concentrations indicated a clearance rate.

$$\log_e C_o - \log_e C_t = \left(\frac{mn}{M} + a \right) * t \quad (3.1)$$

The resulting clearance rates were then corrected to the mass dependent physiological response function using the allometric equation (equation 2.4). Tissue and feces concentrations were normalized to this feeding rate by dividing the concentration on a per gram basis by the clearance rate calculated on a per gram basis yielding a total nmol accumulated per liter of water filtered over the experimental time period. All of the following calculated parameters were completed using the normalized values.

The first parameter I calculated was the amount of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT ingested by the mussels during the feeding period. I defined ingestion as the sum of the total nmol of $^{199}\text{CH}_3\text{Hg}$ or ^{200}HgT in the digestive gland at T_0 plus the total nmol of $^{199}\text{CH}_3\text{Hg}$ or ^{200}HgT in the feces at T_0 normalized to the feeding rate (see the definition in Table 3.1). Adding the T_0 feces to the T_0 digestive gland indicates what was passed through the digestive gland and visceral mass over the course of the feeding experiment and were egested (depurated) by the mussel. Equation 3.2 shows the variables used to calculate ingestion, where DG_{T_0} is the concentration in the digestive gland at T_0 and $feces_{T_0}$ is the concentration in the feces collected from the jars at the conclusion of the feeding period.

$$Ingestion = (DG_{T_0} + Feces_{T_0}) \quad (3.2)$$

Table 3.1-Definitions of calculated parameters.

Term	Definition
Ingestion	This value indicates the total amount of Hg that passed into and through the mussel's digestive gland. Equation 3.2.
Gross Ingestion Efficiency	The ratio of Hg ingested over what was available in the water to indicate what was ingested from the total environment. Equation 3.3.
Absorption Efficiency	The ratio of the concentrations of Hg in the DG to the ingestion value calculated with equation 3.3. This value indicates what was absorbed by the mussel during the 2-hr feeding period. Equation 3.4.
Residual Accumulation (RA)	Ratio of the total concentration in DG to the total concentration depurated at a specific time point. This value indicates the bioavailability of the Hg bound to marine snow.* Equation 3.6.
* This ratio does not account for excretion of metabolic waste which does contribute to overall depuration of a metal species; therefore it is a less conservative estimate of accumulation.	

Although ingestion gives an estimate of how much $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was ingested during the feeding period, it does not indicate the relative amount of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT ingested. Gross ingestion efficiency was used to determine how much $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was ingested from the total of what was spiked into the water. The gross ingestion efficiency was calculated by dividing the total ingestion at T_0 (equation 3.2) by the total nmol of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT spiked into the water (equation 3.3). This ratio accounts for the ingestion of both particulate-bound and dissolved-phase CH_3Hg and HgT .

$$\text{Gross Ingestion Efficiency} = \frac{DG_{T_0} + \text{Feces}_{T_0}}{\text{Water}_{\text{spike}}} * 100 \quad (3.3)$$

Next, absorption efficiency at T_0 was calculated to estimate how much $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was absorbed into the DG during the feeding period. Absorption efficiency was defined as the ratio of the total concentration accumulated in the digestive gland of the mussel during the exposure period to the total ingested (equation 3.4).

$$\text{Absorption Efficiency} = \left(\frac{DG_{T_0}}{DG_{T_0} + \text{Feces}_{T_0}} \right) * 100 \quad (3.4)$$

Although detecting Hg isotope concentrations in the digestive gland potentially indicates uptake from the dissolved or particulate phase, calculating the assimilation efficiency of the metal truly indicates the bioavailability and uptake of the trace metal. Wang and Fisher (1999) define assimilation efficiency

(AE) as the uptake of a contaminant from ingested food minus the amount of contaminant depurated and excreted from the animal. The higher the AE the more bioavailable the compound is to the animal and the more it is likely to bioaccumulate (equation 3.5) (Wang and Fisher, 1999; Wang et al., 1996).

$$AE = (\text{Ingestion} - \text{Excretion} - \text{Egestion}) / \text{Ingestion} \quad (3.5)$$

Wang et al. (1996) and Griscom et al. (2002) assume that loss of heavy metals from excretion is minimal. Excretion in bivalves comprises both mucus excretion and nitrogen waste. Mucus is produced to efficiently move captured particles throughout the mussel's digestive system, beginning at the gill, and is produced in the formation of pseudofeces. In the case of this study, the excretion factor in equation 3.5 was expected to have minimal contribution to the overall loss of CH₃Hg and HgT from the mussels as the major form of excretion in mussels is the nitrogen waste products of protein catabolism. Bayne and Newell (1983) reported that out of a review of a variety of bivalve species, 60% to 90% of nitrogen excretion is composed of ammonia. CH₃Hg and HgT do not readily bind with nitrogen compounds. Another waste product produced by mussels is glutathione. Glutathione is a sulfur-rich protein, used by organisms as a detoxicant of heavy metals. In fact, it is widely used as a biomarker of exposure to pollutants (Clarkson and Magos, 2006; Canesi et al., 1999). Recall, they have a strong binding affinity for sulfur-rich compounds and proteins. In this study, concentrations of glutathione production by the mussels were expected to be low since the Hg concentrations used were not much higher than the environment (unlike Canesi et al. who spiked at 0.2 µM Hg(II) concentrations). Therefore, a spike in glutathione was not expected. Although I expected excretion to be minimal, just like the previously cited authors, I still decided to define the "assimilation efficiencies" calculated as residual accumulation (RA). The residual accumulation for the digestive glands collected at 36 hr and 72 hr was calculated by using equation 3.6. DG refers to digestive gland concentration at a specific time point corrected to the feeding rate, egestion refers to the total concentration in the feces collected at all time-points up to 36 hr or 72 hr and normalized to the feeding rate, and x represents the time point analyzed.

$$RA = \left(\frac{DG_{Tx}}{DG_{Tx} + Egestion_{Total}} \right) * 100 \quad (3.6)$$

Statistical analysis of all data was conducted using SigmaStat 3.5 software. Two-way ANOVA analyses were completed followed by a full post-hoc Tukey test for multiple comparisons. Linear regression analysis was used to compare relationships between each calculated parameter in Table 3.1 (i.e., gross ingestion efficiency, absorption efficiency, etc) and percent Hg incorporation into marine snow. An alpha (α) value of 0.05 was assigned for all tests. Any tests that failed equal variance were \log_{10} transformed.

Results and Discussion

Overall results for incorporation, DOC, log K_d , TSS, gross ingestion efficiency, absorption efficiency, and residual accumulation are presented in Table II.1 included in Appendix II. A summary table of the two-way ANOVA results is included in Table II.2 in Appendix II. Linear regression results of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT are summarized in Table II.3 and II.4, respectively, in Appendix II. Average SRM and RPD values for $^{199}\text{CH}_3\text{Hg}$ analysis were 77% and 12%; respectively. The MDL value for $^{199}\text{CH}_3\text{Hg}$ was calculated using the raw peak areas. The average MDL calculated for $^{199}\text{CH}_3\text{Hg}$ analysis was 21,000 peak areas. Average SRM and RPD values for ^{200}HgT analysis were 89% and 19%. The average MDL for ^{200}HgT analysis was 36 nmol g^{-1} . It is important to note that a total mass balance calculation of CH_3Hg and HgT accounted for ca. 100% of the CH_3Hg and HgT added to the experiments.

$^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT Incorporation into marine snow

Masses of collected marine snow from the rolled treatments and settled particulates from the unrolled treatments were measured for both experiments 1 and 2, which were conducted with water sampled on two different days (see below; Figure 15). Two-way ANOVA analysis, followed by a full Tukey test, was used to compare the masses. No significant interaction was detected ($p > 0.05$) indicating that the mass of marine snow or particulates was not dependent on the treatment nor the experiment.

There was a significant difference between the mass in the rolled and unrolled treatments within each experiment and between each experiment ($p < 0.05$). Significant differences are indicated by different letters above the bars. In the unrolled treatment in experiment 1 two of the three replicates weighed approximately 5mg, but one replicate weighed 2mg. The low, 2-mg outlier was not included in Figure 15.

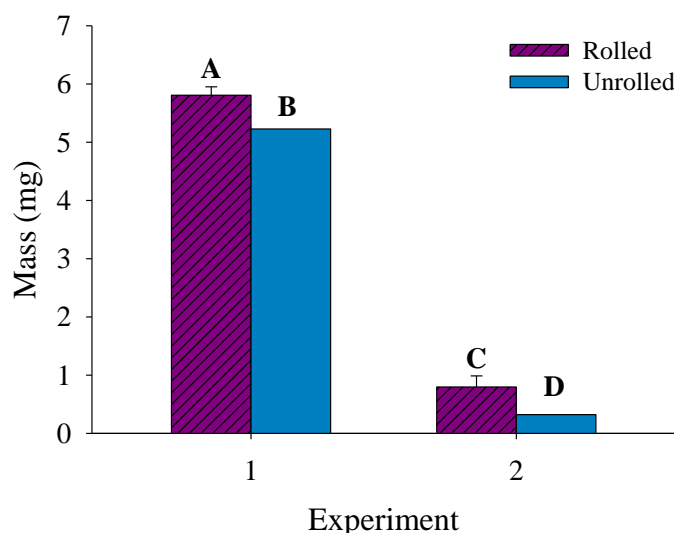


Figure 15- Masses of marine snow collected from rolled treatments and settled particulates collected from unrolled treatments on both experiment days. Two-way ANOVA (followed by Tukey test) analysis determined a significant difference between the rolled and unrolled treatments within each experiment and between each experiment ($p < 0.05$). The data presented in the rolled bars represent the mean \pm SD, $n=3$. The data presented in the unrolled bars represent the mean; $n=2$. One replicate in the unrolled treatment in experiment 1 was significantly lower than the others and was therefore excluded from this figure.

Although there is a significant difference between the mass in the rolled and unrolled treatments within each experiment the averages are still closer than expected. It was anticipated that the mass of the marine snow collected from the rolled jars would be much greater than the settled particulates in the unrolled jars. This indicates that there are still similar quantities of ca. 300- μ m particulates remaining in the water when marine snow is not actively produced by rolling compared to quantity of marine snow produced. Comparison of the amount of marine snow collected and the TSS for these two waters (Table II.1) indicates that a large fraction of the TSS was not aggregated into the marine snow even in the rolled jars. This result was unexpected, but it may explain why the results in Chapter 2 indicate no significant

difference between mussels exposed to ^{200}HgT incorporated into marine snow in the rolled treatments and ^{200}HgT incorporated in particle in the unrolled treatments. In both treatments, there are similar concentrations of bioavailable particulates. The large differences in mass between experiment 1 and 2 eliminated the possibility of pooling the data from both days together.

The incorporation of both isotopes ($^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT) differed significantly over the two day experimental period (see below; Figures 16A and 16B). Incorporation of $^{199}\text{CH}_3\text{Hg}$ ranged from 2% to 28%, whereas incorporation of ^{200}HgT ranged from 0.3% to 17%. Two-way ANOVA analysis of the incorporation of $^{199}\text{CH}_3\text{Hg}$ did not find a significant interaction effect between the treatment (i.e., rolled and unrolled) and the experiment (i.e., 1 and 2). There was a significant difference between the rolled and unrolled treatments in experiment 1 ($p < 0.05$), but no significant difference between the rolled and unrolled treatments in experiment 2 ($p > 0.05$). Additionally, there was a significant difference within the rolled treatments between experiment 1 and 2 and within the unrolled treatments between experiment 1 and 2 ($p < 0.05$). Two-way ANOVA analysis of the incorporation of ^{200}HgT found a significant interaction effect ($p < 0.05$) between the treatment (i.e., rolled and unrolled) and the experiment (i.e., 1 and 2). The interaction effect implies that the amount of ^{200}HgT incorporated into marine snow from the rolled treatment or settled particulates from the unrolled treatment was dependent on the experimental day. This result demonstrates that the seawater composition was significantly different between experiments such that incorporation was limited. Similar to the CH_3Hg results, there was a significant difference between the rolled and unrolled treatments in experiment 1 ($p < 0.05$), but no significant difference between the rolled and unrolled treatments in experiment 2 ($p > 0.05$). Again, there was a significant difference within the rolled treatments between experiment 1 and 2 and a significant difference within the unrolled treatments between experiment 1 and 2. Letters above the bars in the figures indicate significant differences. Those with similar letters are not significantly different, whereas those with different letters are significantly different.

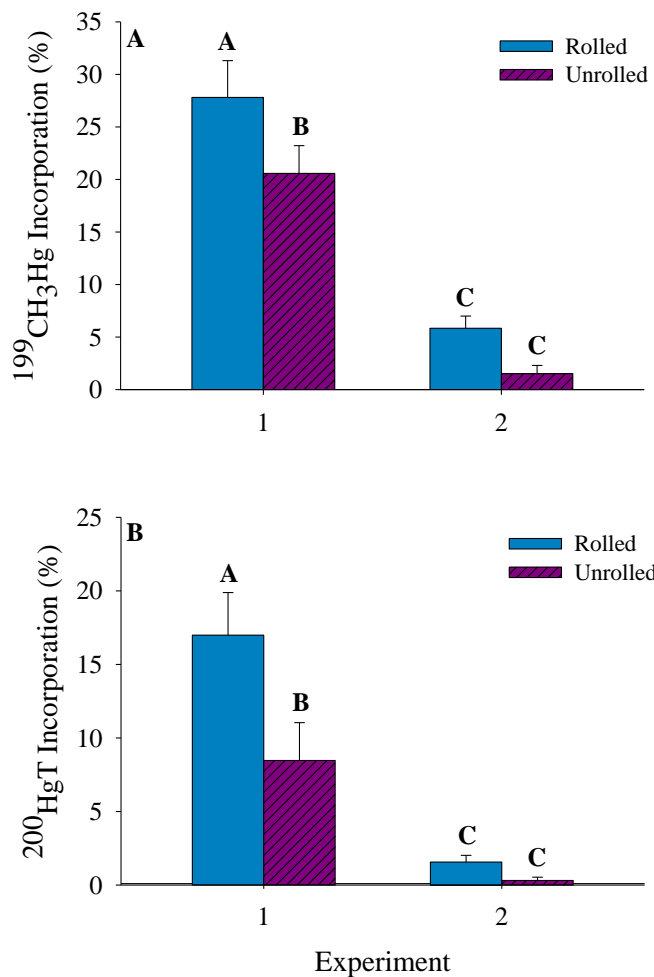


Figure 16- Incorporation of $^{199}\text{CH}_3\text{Hg}$ (A) and ^{200}HgT (B) into collected marine snow versus experiments. Two-way ANOVA (followed by a Tukey test) no significant interaction ($p>0.05$) in between the experiment and $^{199}\text{CH}_3\text{Hg}$, but did find a significant interaction ($p<0.05$) between the experiments and ^{200}HgT incorporation. There was as significant difference between the rolled and unrolled treatments in experiment 1, but no difference between rolled and unrolled treatments in experiment 2. Bars with similar letters are not significantly different, those with different letters are. Data represents the mean \pm SD; $n=3$.

In each treatment and in each experiment, more $^{199}\text{CH}_3\text{Hg}$ was incorporated into the collected marine snow than ^{200}HgT which was the opposite result of what is presented in Chapter 2 (Figure 9A and 9B). Although more $^{199}\text{CH}_3\text{Hg}$ was incorporated into the marine snow than ^{200}HgT the incorporation percentages were lower than expected, but were similar to those presented in Chapter 2. Similarly to the results in Chapter 2, there was a significant difference between the incorporation of both forms of Hg in the rolled and unrolled treatments. It was anticipated that the incorporation in the unrolled treatments

would be close to zero, similar to Chapter 2, but rather, the incorporation is higher than zero and closer to the incorporation when marine snow was produced. The mass of the particulates collected from the unrolled treatments explains this trend (Figure 15). Since there was a relatively high mass of 300- μm particulates collected in the unrolled treatment, more incorporation should be expected to occur since $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT have high partition coefficients ($\log K_d$ ca. 5 L kg^{-1}) and are particle reactive and would accumulate in the smaller particles as well. Again, the difference in TSS and marine snow collected indicates a higher concentration of particles remaining in suspension that were not incorporated into the marine snow or collected.

The partition coefficients ($\log K_d$) can be used as a proxy for the propensity of an element to be associated with the solid phase. The K_d for Hg(II) is several orders of magnitude higher than other metals, and typically higher than that of CH_3Hg . The calculated partition coefficients ($\log K_d$) for $^{199}\text{CH}_3\text{Hg}$ were $4.7 \pm 0.09 \text{ L kg}^{-1}$ and $4.8 \pm 0.1 \text{ L kg}^{-1}$ for the unrolled and rolled treatments in experiment 1 and $4.7 \pm 0.1 \text{ L kg}^{-1}$ and $4.9 \pm 0.04 \text{ L kg}^{-1}$ for the unrolled and rolled treatments in experiment 2, respectively. The calculated partition coefficients for ^{200}HgT were $4.4 \pm 0.1 \text{ L kg}^{-1}$ and $4.5 \pm 0.02 \text{ L kg}^{-1}$ for the unrolled and rolled treatments in experiment 1 and $4.0 \pm 0.1 \text{ L kg}^{-1}$ and $4.3 \pm 0.1 \text{ L kg}^{-1}$ in the unrolled and rolled treatments in experiment 2, respectively. These calculated values are lower than previously published values for Hg partitioning onto inorganic materials or suspended solids in natural waters ($\log K_d > 10^5 \text{ L kg}^{-1}$ typically) (Stordal et al, 1996). The $\log K_d$ values were lower for ^{200}HgT than for $^{199}\text{CH}_3\text{Hg}$, again this may reflect higher concentrations of organic matter than inorganic matter in the marine snow. The higher $\log K_d$ for $^{199}\text{CH}_3\text{Hg}$ indicates that more $^{199}\text{CH}_3\text{Hg}$ was associated with the collected marine snow and settled particulates than was remaining in the seawater (dissolved and suspended) after sampling. Although these results suggest more CH_3Hg was incorporated into the collected marine snow and settled particulates than ^{200}HgT they also suggest that the opposite is true for the uncollected particulates. A lower $\log K_d$ value most likely indicates that less ^{200}HgT was bound to the solid phase that was collected. Many particulates remain in the water and were not collected after the initial marine snow was removed from the rolled jars. All of the remaining particulates $<300\mu\text{m}$ down to $6\mu\text{m}$ are still bioavailable for the

mussels and were potential binding sites for $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT . It would appear that more ^{200}HgT remained in suspension after particle sampling and was bound to smaller particulates that had not settled to the bottom of the jars. The data in Appendix I tend to support this assertion for the unrolled treatments, especially as the K_d is similar for the 0.2- to 8- μm fraction and the collected aggregate fraction. If surface area was the determining factor the K_d would be greatest for the smaller particles, but the data presented in this chapter and Appendix I suggests the binding is dependent on the nature of the material that forms each fraction. As previously stated, 15 minutes of settling time only allows $\geq 300\ \mu\text{m}$ marine snow to accumulate on the bottom of the jars for collection.

The significant difference of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT incorporation between experiments can also be related to the chemical composition of the seawater used. The production of marine snow is dependent on the presence of both organic and inorganic materials. Inorganic particulates are required to serve a nucleation sites that organic matter can stick to such that as particles collide they coagulate to form larger particulates. Organic matter is required for cohesion of particles. If there is less organic matter less coagulation of particulates can occur because they are lacking the “glue” to cause them to stick. The organic matter also provides binding sites for trace metals, such as $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT . If there is less organic matter it is less likely that there are sulfur-compounds present for binding. It is important to note that prior to collection for experiment 2, a brief thunderstorm occurred in the area. This rain event had high winds and strong rainfall causing re-suspension of sediments into the water column. The salinity at the beginning of experiment 1 was 26.6‰ and the measured salinity at the beginning of experiment 2 was 28.1‰. The total suspended solids, after sieving through a 100 μm mesh was 26 mg L^{-1} for experiment 1 and 20 mg L^{-1} for experiment 2; these values were not significantly different. The dissolved organic carbon (DOC) concentrations were 173 μM for experiment 1 and 145 μM for experiment 2. The slight decrease in DOC may indicate a decrease in overall organic matter in the water. The turbulent weather that occurred the morning of experiment 2 may have caused an increased concentration of inorganic materials relative to organic materials that limited both the mass of marine snow produced and the incorporation of both forms of Hg. Marine snow was observed forming within 1 hr after rolling began

which was much faster than previously observed in any experiment, but these may have been large re-suspended, inorganic particles (i.e., clays and sediments) that were collected and passed through the sieve.

In summary, it would appear that incorporation of both $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was lower than expected in the rolled treatments (Figures 16A and 16B). This is likely due to the presence of suspended particles ($<300\text{ }\mu\text{m}$) remaining in the water after the marine snow was collected. These suspended particles potentially served as additional binding sites for $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT . I did not collect these particles, therefore, I cannot account for the additional partitioning of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT from solution. It would appear that the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT remained in the water column based on my sampling techniques, but in fact, it may be that the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT were particle-bound. Various size fractions ($\geq 300\text{ }\mu\text{m}$, 8- to $300\text{ }\mu\text{m}$, and $0.2\text{ to }8\text{ }\mu\text{m}$) should be collected via filtration at the conclusion of rolling experiments in order to account for all bioavailable particles in suspension. A particulate fraction experiment was conducted to observe the partitioning of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT to different size fractions (see Appendix I) and the results of this study tend to confirm the discussion above.

Ingestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT by blue mussels

Ingestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was determined by adding the total nmol of $^{199}\text{CH}_3\text{Hg}$ or ^{200}HgT in the digestive gland at T_0 and the total nmol of $^{199}\text{CH}_3\text{Hg}$ or ^{200}HgT in the feces collected after the feeding period (equation 3.2). The average ingestion of $^{199}\text{CH}_3\text{Hg}$ was $0.4 \pm 0.4\text{ nmol}$ and $0.6 \pm 0.5\text{ nmol}$ in the unrolled and rolled treatments in experiment 1 (respectively) and $0.5 \pm 0.4\text{ nmol}$ and $0.9 \pm 0.1\text{ nmol}$ in the unrolled and rolled treatments in experiment 2 (respectively). The average ingestion of ^{200}HgT was $0.9 \pm 0.8\text{ nmol}$ and $2.0 \pm 0.7\text{ nmol}$ in the unrolled and rolled treatments in experiment 1 (respectively) and $3.2 \pm 3.2\text{ nmol}$ and $6.8 \pm 3.3\text{ nmol}$ in the unrolled and rolled treatments in experiment 2 (respectively).

Gross ingestion was calculated by taking the total ingested and dividing those values by the total nmol of $^{199}\text{CH}_3\text{Hg}$ or ^{200}HgT spiked into the water (equation 3.3). These values indicate the gross

ingestion from what was added to the water during the feeding period. Linear regression analysis of the gross ingestion of $^{199}\text{CH}_3\text{Hg}$ versus the percent $^{199}\text{CH}_3\text{Hg}$ incorporated into marine snow yielded no significant relationship ($p>0.05$) (see below; Figure 17A). Linear regression analysis of the gross ingestion of ^{200}HgT versus the percent ^{200}HgT incorporated into marine snow yielded also no significant relationship ($p>0.05$) (see below; Figure 17B).

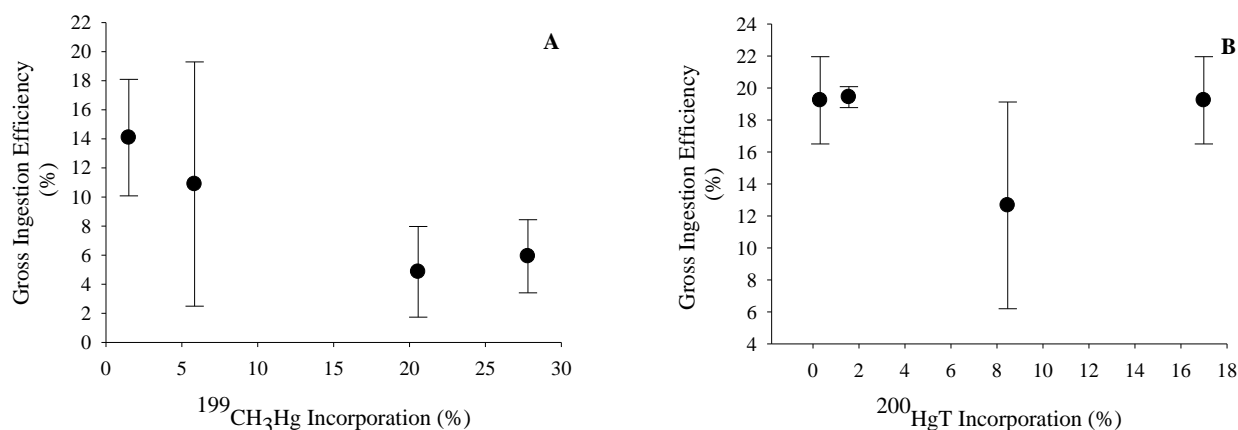


Figure 17-Gross ingestion efficiency of $^{199}\text{CH}_3\text{Hg}$ (A) and ^{200}HgT (B) versus their respective percent incorporations into marine snow. Linear regression analysis of both relationships yielded no significant relationship ($p>0.05$). Data represents the mean \pm SD; $n=3$.

I had anticipated a positive linear correlation between the gross ingestion of either form of Hg and the incorporation into marine snow and particulates. That is, I expected that more incorporation into marine snow would increase the bioavailability of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT . However, linear regression analysis did not determine a significant relationship between gross ingestion of $^{199}\text{CH}_3\text{Hg}$ and $^{199}\text{CH}_3\text{Hg}$ incorporation into marine snow. There appears to be a slight elevation in the $^{199}\text{CH}_3\text{Hg}$ gross ingestion at lower $^{199}\text{CH}_3\text{Hg}$ incorporation into marine snow (Figure 17A). This result may indicate that the mussels were able to ingest more $^{199}\text{CH}_3\text{Hg}$ when less was incorporated into the marine snow. Another possibility is that the mussels were actively rejecting the marine snow that had more $^{199}\text{CH}_3\text{Hg}$ incorporated. The data presented in Figure 17B also shows no trend between the gross ingestion of ^{200}HgT and ^{200}HgT incorporated into marine snow. Note that the gross ingestion efficiencies on the y-axes demonstrate that the mussels were ingesting both forms of Hg, but the amount ingested was not dependent on the amount

in the marine snow. Instead, the mussels may have also ingested $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT from other particulates in suspension rather than from the marine snow. Again, we go back to the hypothesis from the previous chapter that there may be bioavailable particulates bound with $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT remaining in suspension after the formation of marine snow.

Next, I wanted to examine the amount of Hg ingested by the mussel from what was physically bound in the marine snow versus the amount incorporated into the marine snow. The ingestion values previously calculated (equation 3.2) were divided by the total nmol $^{199}\text{CH}_3\text{Hg}$ and total nmol ^{200}HgT in the marine snow (equation 3.7).

$$\frac{[DG_{T0} + Feces_{T0}]}{[Marine\ snow]} \quad (3.7)$$

This ratio indicates whether or not what the mussels ingest was from the marine snow. A lower ratio value would indicate an increased uptake from the marine snow, whereas a higher ratio value would indicate ingestion from particles other than marine snow. The calculated ratios were plotted against the percent incorporated into the marine snow (see below; Figures 18A and 18B). Linear regression analysis of both Figure 18A and 18B yielded no significant correlation ($p < 0.05$).

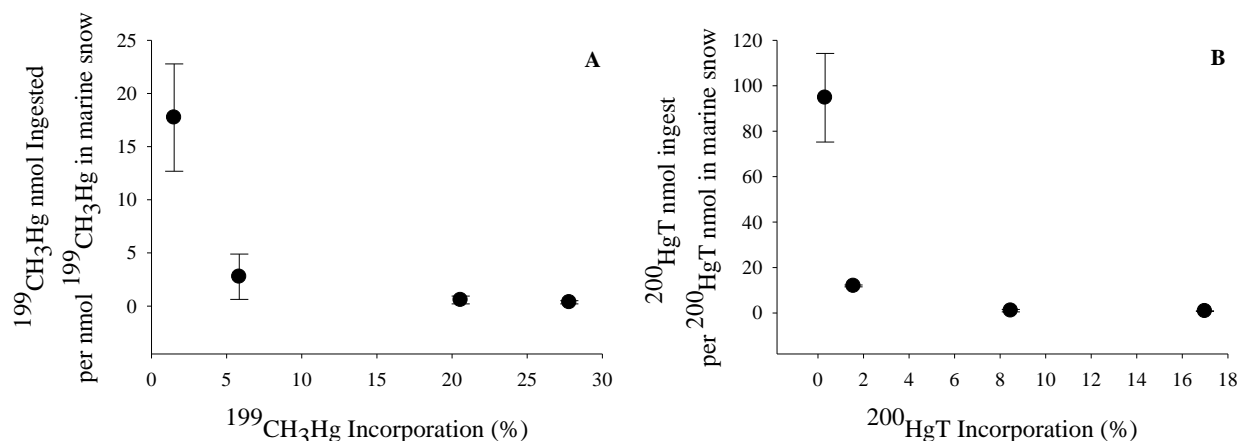


Figure 18-The ratio of the total nmol of $^{199}\text{CH}_3\text{Hg}$ (A) and ^{200}HgT (B) ingested to the total nmol of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT incorporated into the marine snow versus the percent $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT incorporated into the marine snow. Linear regression analysis indicated no significant relationship ($p>0.05$) for either $^{199}\text{CH}_3\text{Hg}$ or ^{200}HgT . Data represent the mean \pm SD; n=3.

Both of these figures show that ingestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT did not depend on how much was in the marine snow. Again, it would appear that less is ingested as incorporation into the marine snow increased. The lack of ingestion with increased incorporation may imply that the mussels were rejecting the marine snow as more was incorporated, which is opposite of what I hypothesized. Although unexpected, these results would agree with the conclusions drawn by McAloon and Mason (2003) and Lawrence et al. (1999). Both reported decreased bioavailability of Hg as organic matter increased. If my theory that incorporation in experiment 1 was greater than experiment 2 because of an increased concentration of organic matter, then these ingestion results would confirm that the increase in organic matter decreased the availability of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT to the mussels.

Absorption and Accumulation of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT by blue mussels

Absorption efficiencies were calculated for mussels immediately after exposure (T_0) to indicate the absorption of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT (Figure 19A and 19B) during the feeding period (equation 3.4). Linear regression analysis of $^{199}\text{CH}_3\text{Hg}$ absorption efficiency versus $^{199}\text{CH}_3\text{Hg}$ incorporation into

collected marine snow yielded no significant relationship ($p>0.05$). Linear regression of ^{200}HgT absorption efficiency versus ^{200}HgT incorporation into collected marine snow yielded no significant relationship ($p>0.05$). The first three $^{199}\text{CH}_3\text{Hg}$ data points are an average of two values while the last data point is an average of three values with error bars representing the standard deviation. Each of the first three points had a high outlier (100%) because either the mussel did not produce feces or the feces had no detectable $^{199}\text{CH}_3\text{Hg}$. The third ^{200}HgT data point also had a high outlier due to a zero concentration value in the feces; therefore, I considered these values to be inaccurate so these values were removed.

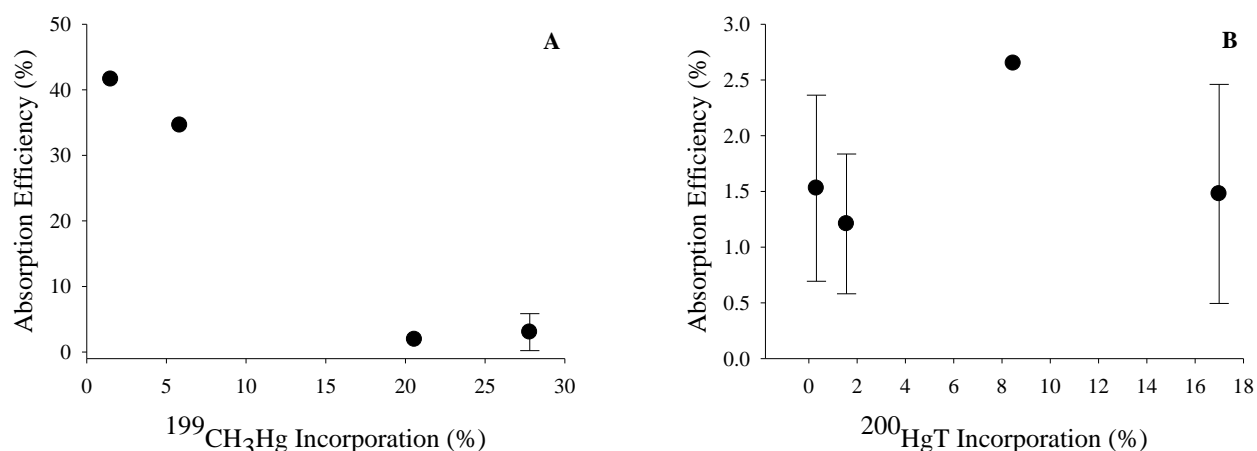


Figure 19- Absorption efficiency of $^{199}\text{CH}_3\text{Hg}$ (A) and ^{200}HgT (B) versus percent incorporated into collected marine snow. Linear regression analysis of both $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT yielded no significant relationship ($p>0.05$). Data points without error bars represent an average of two values due to outliers that were removed. Those with error bars represent an average of three values with the standard deviation.

A greater absorption efficiency value on the y-axes indicates low concentrations of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT in the feces collected from the feeding jars at the end of the feeding period, but high concentrations of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT in the digestive gland at the end of the feeding period (see equation 3.4). I had expected to see an increasing trend in absorption efficiency as the incorporation into marine snow increased, but rather my data shows no trend for either form of Hg.

The majority of the data points in Figure 19A are close to zero, but the mussels exposed to the lowest incorporation appear to have an elevated absorption of $^{199}\text{CH}_3\text{Hg}$. This may demonstrate that it was easier for the mussels to ingest the $^{199}\text{CH}_3\text{Hg}$ from marine snow with less $^{199}\text{CH}_3\text{Hg}$ incorporated, or the mussels were ingesting $^{199}\text{CH}_3\text{Hg}$ from other particulates in the water other than the marine snow similar to the results in Figure 18. The low absorption efficiency of $^{199}\text{CH}_3\text{Hg}$ as incorporation increased may also indicate possible rejection of the marine snow by the mussels. Once $^{199}\text{CH}_3\text{Hg}$ was incorporated into the marine snow the marine snow may have been too difficult for the mussels to break apart on the labial palps for ingestion. This data implies that increased incorporation in marine snow decreased the bioavailability of $^{199}\text{CH}_3\text{Hg}$.

Figure 19B shows no significant trend between absorption efficiency and incorporation of ^{200}HgT into marine snow ($p>0.05$). Closer examination of the absorption efficiency values on the y-axis shows a much lower absorption efficiency of ^{200}HgT than $^{199}\text{CH}_3\text{Hg}$ which agrees with the literature (Wang et al., 2004; McAloon and Mason, 2003; Gagnon and Fisher, 1997). Previous studies reported low absorption and assimilation of inorganic mercury (HgT) in comparison to $^{199}\text{CH}_3\text{Hg}$. Bivalves have been observed to depurate (in the form of feces) the majority of HgT after exposure while depurating less CH_3Hg (Wang et al., 1995). Therefore, although I expected to see low absorption of ^{200}HgT in comparison to $^{199}\text{CH}_3\text{Hg}$, I still expected to see a positive linear trend with increasing incorporation. Again, these results imply rejection of contaminated marine snow.

Although the lack of correlation between $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT absorption versus incorporation implies possible rejection of the marine snow by the mussels, it does not confirm either rejection or assimilation. Residual accumulations (RA) were calculated for mussels sacrificed at T_{36} and T_{72} (equation 3.6). Elevated concentrations of Hg in the digestive glands from these time points are true representations of accumulation because after at least 36 hours most of the Hg has been depurated by the mussel. Published studies of the depuration of trace metals by mussels have shown gut residence times of <36 hr (Griscom et al, 2002; Boening, 1997; Wang et al., 1995). Therefore, it is expected that any Hg remaining in the digestive gland at this time point has been intracellularly digested and assimilated by the mussel.

Average residual accumulation values were calculated for mussels sacrificed at T₃₆ and T₇₂, but due to high variability the 72-hr data was not included in this report. Linear regression analysis of the residual accumulation ¹⁹⁹CH₃Hg in the mussels at 36 hr versus the incorporation of ¹⁹⁹CH₃Hg in marine snow yielded no significant correlation (p>0.05). Linear regression analysis of the residual accumulation of ²⁰⁰HgT in the mussels at 36 hr versus the incorporation of ²⁰⁰HgT in marine snow also yielded no significant correlation (p>0.05).

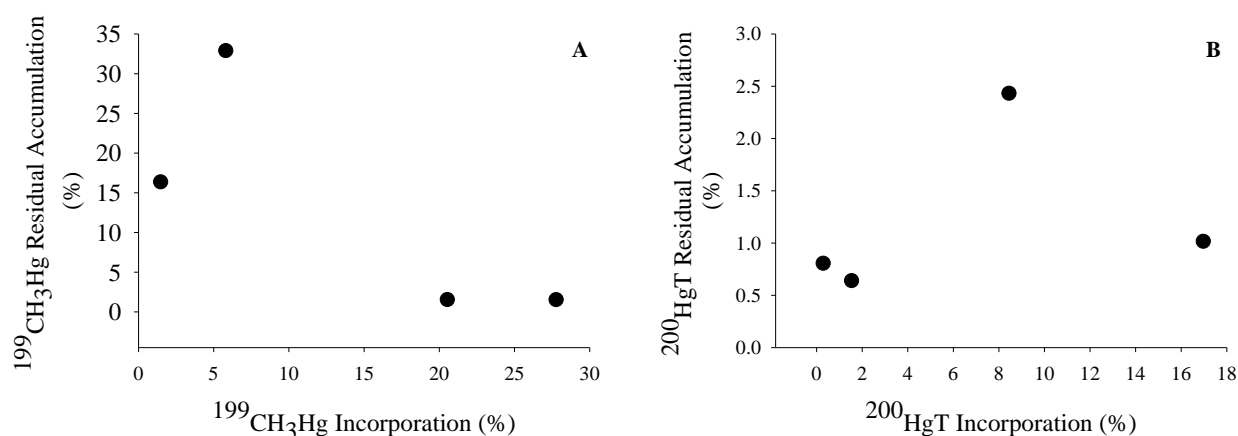


Figure 20- Residual accumulation of ¹⁹⁹CH₃Hg (A) and ²⁰⁰HgT (B) after 36 hr versus percent incorporated into marine snow. Linear regression analyses of both forms of Hg yielded no significant relationship (p>0.05). Data represent the mean; n=2.

Figures 20A and 20B show no significant relationship between the residual accumulations of either form of Hg in mussels sacrificed at T₃₆ and the amount of ¹⁹⁹CH₃Hg and ²⁰⁰HgT incorporated into marine snow. These data confirm that the amount of ¹⁹⁹CH₃Hg and ²⁰⁰HgT accumulated in mussels was not dependent upon the amount of ¹⁹⁹CH₃Hg and ²⁰⁰HgT incorporated into the marine snow. There appears to be a slight elevation in accumulation at low incorporation in the ¹⁹⁹CH₃Hg exposures again indicating uptake of ¹⁹⁹CH₃Hg from other particles besides marine snow or the possible rejection of contaminated marine snow as incorporation increased. Similarly to the previous graphs, ¹⁹⁹CH₃Hg accumulation was much greater (30% maximum) than HgT accumulation (1.5% maximum). The

differences in accumulation demonstrate the difference in bioavailability. HgT was less bioavailable than $^{199}\text{CH}_3\text{Hg}$ in marine snow.

Although the results show no significant relationship between absorption and accumulation versus incorporation, there was an observable decrease in absorption and accumulation of $^{199}\text{CH}_3\text{Hg}$ as incorporation increased. This may be due to a decrease in bioavailability of the $^{199}\text{CH}_3\text{Hg}$ as organic matter increased as reflected in the DOC concentrations. McAloon and Mason (2003) and Lawrence et al. (1999) reported a decrease in the uptake of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT by deposit feeding amphipods as the percent organic matter increased in the sediment. The decrease in bioavailability as organic matter increases may be directly related to the binding capacity of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT for particulates (high $\log K_d$). If the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT formed strong bonds with the materials in the marine snow the pH of the gut may not have been low enough to solubilize the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT from the particulates. It also may be possible that the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT bound so tightly within the marine snow that it caused the marine snow to become compact to the point that the mussels could not shear the marine snow apart on the labial palps. If the marine snow could not be broken down into smaller particles for the mussel to ingest then the mussel would reject the marine snow in pseudofeces. This theory would explain why the mussels could have been ingesting smaller particles bound with $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT rather than the marine snow, but the concentration of CH_3Hg spiked was low enough that it I do not expect it to affect binding within the marine snow. Again, the other possibility for rejection is that the rolling process itself created particles that were tightly coagulated.

These reported residual accumulation values of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT at T_{36} are comparable to the literature. For example, Gagnon and Fisher (1997) reported assimilation efficiencies (AE) ranging from 1 to 9% for HgT and 30 to 88% for CH_3Hg depending on the organic content of sediments fed to mussels. A similar absorption efficiency study by Wang et al., (2004) reported AE's ranging from 17 to 66% for HgT and 53 to 90% for CH_3Hg for mussels fed plankton that were exposed to mixtures of Hg and Se. Both of these studies chose to disregard the excretion factor of the AE calculation (presented in equation

3.6); therefore our results are comparable to the results presented by Gagnon and Fisher (1997), but less than those published by Wang et al. (2004).

The lack of significant correlations between the accumulation and incorporation may also be due to the methodology to produce marine snow. The published studies aforementioned utilized specific food concentrations and properties (i.e., chemical properties of sediments or type of phytoplankton) in a more controlled environment compared to this study. Gagnon and Fisher (1997) used particulates that were manufactured to be uniform in size and content while Wang et al., (2004) cultured specific phytoplankton species. The production of marine snow from raw seawater collected from the environment is a source of variability in the experimental methods. As evident by Figures 15 and 16, the masses and incorporation differed between each day due to changes in the seawater composition; therefore, there was less control over experimental parameters.

Egestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT

Results of feces sample analyses were used to compare the amount of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT depurated over time. Both $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT concentrations in the feces collected decreased over time. These results show that the mussels were able to eliminate $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT from their bodies after ingesting contaminated marine snow. Depuration of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT serves as a proxy for ingestion; therefore, these data confirm that the mussels ingested some of the contaminated marine snow. By T_{36} most of the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT has been depurated, again indicating a gut residence time of 36 hr. There is no significant difference between the concentrations in the feces and exposure in both Figures 21A and 21B. Therefore, it can be concluded that the mussels ingested both forms of Hg with or without marine snow.

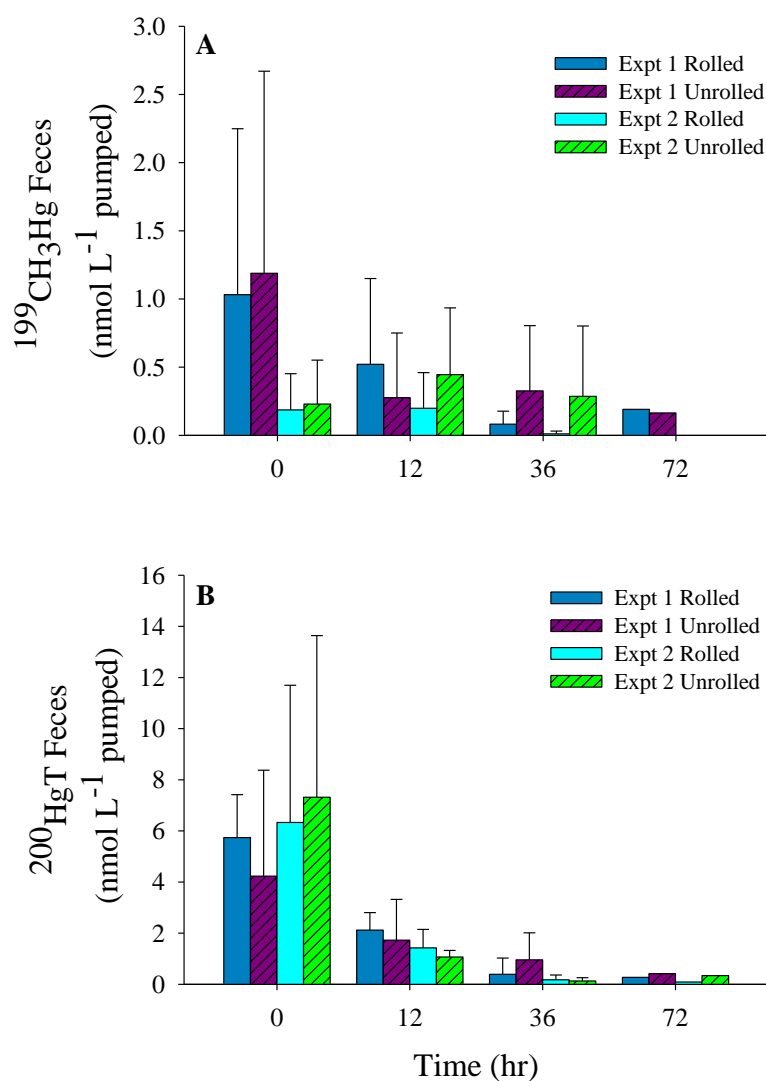


Figure 21- Depuration of $^{199}\text{CH}_3\text{Hg}$ (A) and ^{200}HgT (B) over time after the feeding period. Data represents the mean \pm SD except for the T_{72} samples (T_0 , n=8; T_{12} , n=6; T_{36} , n=4; T_{72} , n=2).

Close inspection of the concentration of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT in the feces shows an order of magnitude difference between both forms of Hg. As expected there was more ^{200}HgT in the feces than $^{199}\text{CH}_3\text{Hg}$. There should be more HgT in the feces since it is less bioavailable and more rapidly depurated than CH_3Hg . Recall that $^{199}\text{CH}_3\text{Hg}$ is more readily soluble at lower pH and is more readily absorbed (McAloon and Mason, 2003). Also recall that the average gut pH in *Mytilus edulis* is ca. 5.6. Therefore, I expected to see less $^{199}\text{CH}_3\text{Hg}$ depurated than HgT because more should have been solubilized in the

digestive gland and phagocytosed. The increased depuration of HgT agrees with the absorption and accumulation data which was lower than $^{199}\text{CH}_3\text{Hg}$ (Figures 19 and 20).

Conclusions

In conclusion, both isotopes of Hg were successfully incorporated into marine snow, but ingestion of the isotopes was not dependent on the presence of marine snow. I hypothesize that ingestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was independent of incorporation into marine snow because there was a large concentration of bioavailable particulates ($<300\ \mu\text{m}$, $>6\ \mu\text{m}$) remaining in suspension after the production of marine snow. These particulates created an artifact in the data such that mussels in the unrolled treatments ingested as much particulate-bound $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT as the mussels in the rolled treatments. Additionally, mussels absorbed and accumulated $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT in low incorporation exposures due to the presence of these particulates. It may be that the smaller particulates were easier for the mussels to ingest than the marine snow resulting in the mussels rejecting the marine snow. The marine snow may have been difficult to ingest due to physico-chemical binding between $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT and the organic matter within the marine snow.

Particulates remaining in the water after marine snow was formed may have created an artifact in the feeding experiment. Since mussels can ingest particulates as small as $6\ \mu\text{m}$ in size, then it is likely the mussels were exposed to particulate bound $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT regardless of the presence of marine snow. It had been assumed that production of marine snow coagulated all of the suspended particulates in the water when rolled, but the data suggests otherwise. Recall that in the feeding experiments the mussels were directly placed into the jars filled with laboratory produced marine snow. Therefore, they were being exposed to a wide range of particulates potentially bound with $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT other than produced marine snow.

Gross ingestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT was not found to be dependent on the amount of Hg incorporated into marine snow (Figures 17A and 17B). Likewise, the ingestion of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT based on what was available to ingest from the marine snow was not dependent upon incorporation either

(Figures 18A and 18B). This repeating trend (or lack of a trend, thereof) confirms that the incorporation into larger aggregates of either form of Hg does not necessarily increase the bioavailability. In fact, it appears that as more was incorporated less was ingested. This result implies that the mussels may have been actively rejecting the contaminated marine snow because it may have been too difficult for the mussels to break apart on their sorting organ, the labial palps.

Absorption of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT after the feeding experiment was also found to be independent of incorporation into marine snow. The results presented in Figures 19A and 19B show that most of the CH_3Hg and HgT was not absorbed into the digestive gland during the feeding period, but rather were depurated in the feces produced, again implying rejection of marine snow. Finally, Figures 20A and 20B confirm that residual accumulation was independent of incorporation. There was a slight elevation in accumulation at lower incorporation indicating that the mussels were accumulating CH_3Hg , but likely from other bioavailable particulates.

Depuration of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT decreased (Figures 21A and 21B) with time agreeing with widely published results that report that mussels rapidly depurate metals (Blackmore and Wang, 2004; Wang et al., 1995). Most importantly, the depuration of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT serves as a proxy for ingestion, confirming that the mussels did ingest some of the $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT , but it appears that it did not depend on the presence of marine snow.

These results indicate that the role of marine snow may be less important than hypothesized and that the uptake of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT bound to small particles is a more important pathway of Hg uptake. The small particulate interference experienced in this experiment may be eliminated by filtering produced marine snow from rolled jars and transferring it to 0.2- μm filtered seawater in which mussels can be added to feed. The marine snow could even be removed from certain jars such that chosen mussels are only exposed to the particles remaining after the production of marine snow while others are exposed to all of the particulates including marine snow. The main limitation of this design is that marine snow is very delicate and difficult to handle without causing any damage to the structural integrity of the particulates. A second method would be to artificially produce marine snow by using artificial seawater

and spiking it with TEP, organic matter, and inorganic matter. Natural variability would be eliminated and perhaps the trends would be clarified and confirmed. A final possibility would be to pair a size fractionation experiment with a feeding experiment. In order to test my hypothesis of the presence of smaller, bioavailable particulates, I conducted an additional study to determine the distribution of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT amongst various size fractions. (See Appendix I). Although a size fractionation experiment is feasible when conducting a rolling experiment (see Appendix I), it is difficult to execute for feeding experiments.

Chapter 4-Summary of Conclusions and Future Work

Summary of Conclusions

Chapter 2-Quantifying incorporation of CH₃Hg and Hg(II) into marine snow over time and partitioning of Hg in individual mussel tissues

In the first portion of Chapter 2, I conclude that both CH₃Hg and HgT were incorporated into laboratory produced marine snow, thereby confirming my hypothesis that both forms of Hg could be incorporated into marine snow. These results demonstrated that it is probable that both CH₃Hg and HgT are incorporated into settling marine snow in the open environment. A potential artifact of the experimentation is the presence of bioavailable particulates remaining in suspension after marine snow is produced. I suggest sieving the collected seawater through a finer mesh (100 µm) to remove larger plankton, but preserve enough organic and inorganic materials to create marine snow.

In the second portion of Chapter 2, I conclude that the mussels ingested and initially absorbed CH₃Hg and HgT from marine snow in the rolled treatment and from bioavailable particulates in the unrolled treatment. I further supported this conclusion by detailing the presence of CH₃Hg and HgT in the collected feces and digestive glands indicating ingestion had occurred. Tissue partitioning was not confirmed due to high background concentrations. Overall, I concluded that my original hypothesis that marine snow can serve as a pathway for Hg uptake was potentially confirmed with this preliminary data. Finally, I suggest conducting another feeding experiment, but using stable Hg isotopes, ¹⁹⁹CH₃Hg and ²⁰⁰HgT, as tracers such that more mussels could be simultaneously exposed to both forms of Hg and to detect changes in CH₃Hg and HgT over background concentrations.

Chapter 3- Quantifying assimilation of ¹⁹⁹CH₃Hg and ²⁰⁰HgT stable isotopes from marine snow in blue mussels

In Chapter 3 I concluded that ¹⁹⁹CH₃Hg and ²⁰⁰HgT were successfully incorporated into marine snow, but were also incorporated into bioavailable particulates that were not analyzed. Not accounting for these particulates limited my understanding of the incorporation of ¹⁹⁹CH₃Hg and ²⁰⁰HgT and its potential

bioavailability to the mussels. I also concluded that while some ingestion of CH_3Hg and HgT from marine snow occurred, little was assimilated; therefore, my hypothesis that marine snow could act as a potential pathway is rejected.

Overall, I found that ingestion, absorption, and accumulation of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT were independent of the amount of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT incorporated into marine snow. I concluded that this was due to the presence of the bioavailable particulates present after marine snow production (in the rolled treatment) and present freely in suspension (in the unrolled treatment). The mussels were still ingesting and accumulating $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT due to these particulates, even if they were not accumulating $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT from marine snow. In fact, the data presented imply that the mussels may have been rejecting the marine snow and ingesting the smaller particles because they were easier to ingest. These results demonstrated that the role of marine snow as a pathway for $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT uptake may be less important than the role of smaller particulates. Finally, I suggest conducting a particle fractionation experiment to determine the distribution of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT amongst various particle sizes.

Future Work

To continue this study, experiments should be conducted using stable Hg isotopes. The use of these tracers made for easier sampling and clearer results, albeit complicated analytical techniques are required. It would be recommended to conduct several incorporation experiments that include size fractionation (as conducted in Appendix I) such that marine snow ($\geq 300\text{ }\mu\text{m}$), 8- to $300\text{-}\mu\text{m}$ particles (100% bioavailable to mussels), 0.2- to $8\text{-}\mu\text{m}$ particles, and, finally, the dissolved fraction (water) are collected. Comparing the incorporation of Hg into all of these fractions will elucidate the distribution of Hg such that it can be determined which particle size is the most important pathway of uptake for mussels.

Future feeding assays should be conducted with more mussels for more robust statistics, but will require several days to feed more animals; therefore, sources of water and chemical characterization will be important. As evident of the data in the Chapter 3, collecting water on two different days resulted in significantly different incorporation percentages. It may be possible to use the same water sample over the course of a couple of days, but the water would need to be frozen in a glass container to maintain the cleanest environment and prevent major changes in DOC concentration. First, samples of water should be tested to see if marine snow is produced after the water has been frozen.

A field experiment could be conducted to either confirm or nullify the results presented in this thesis. Mussels could be collected from environments where there is high marine snow particle production and where there is low marine snow production. As evident by the data presented in Chapter 2, analysis of separate tissues is not critical. Therefore, whole body masses could be analyzed intact. If marine snow does, in fact, decrease the bioavailability of Hg then the total body burden of the mussels in the high marine snow production environment should be lower than mussels in low marine snow production environments.

Lastly, if the rolling process itself created marine snow that was too compact for mussels to ingest it may prove useful to conduct rolling experiments with larger vessels, shorter rolling periods, or slower rolling speeds. Various experiments have been conducted to produce marine snow. Those that use larger vessels and roll at slower speeds tend to produce lighter, flocculent marine snow (Lyons et al., 2010;

Shanks and Edmondson, 1989). This type of marine snow may be easier for the mussels to digest extra- and intracellularly.

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Appendix I- Determination of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT distribution in three particulate size fractions

Introduction

The results presented in Chapter 2 and 3 indicate that uptake of Hg from marine snow is potentially less important than uptake from smaller, bioavailable particles. I hypothesize that these small particulates may be concentrated enough to bind a large fraction of Hg present in solution and large enough for mussels to capture. If this hypothesis is correct it would explain the lack of significant differences in uptake between rolled and unrolled treatments presented in the previous chapters. It is likely that the mussels were ingesting particulates bound with HgT in the unrolled treatments in the similar proportions to the mussels exposed to marine snow bound with HgT in the rolled treatments.

This follow-up experiment was conducted to determine particulate size fractionation and Hg distribution between the rolled and unrolled treatments. As previously mentioned, mussels can capture particles $\geq 6\ \mu\text{m}$ with ca. 100% efficiency. Therefore, it is important to know how many particles of this size range were not incorporated into the marine snow and were bound with HgT. By determining the concentration of HgT partitioned into the marine snow ($\geq 300\ \mu\text{m}$), 8- to 300- μm particles, 0.2- to 8- μm particles, and the dissolved fraction in the water I was able to calculate a mass balance and determine what fraction of CH_3Hg and HgT the mussels were likely exposed to in the feeding experiments.

I hypothesized that the incorporation of ^{200}HgT would be greatest in the marine snow collected from the rolled treatments in comparison to the incorporation into the 8- to 300- μm particles from the same jars. I also hypothesized, based on my previous data, that incorporation would be significantly greater in the 8- to 300- μm particles than the other particle fractions in the unrolled treatment. If confirmed, my results would explain the unexpected trends I presented in the previous chapters.

Materials and Methods

The materials for this experiment consisted of trace-metal clean 1-L jars, 15-mL polyethylene Falcon tubes, pre-weighed 0.2- and 8- μ m polycarbonate filters, 25-mm frit filter, graduated filter towers, pre-ashed QF/F and GF/F filters, and 250-mL ICHEM bottles.

Seawater was collected from UCONN Avery Point beach (41° 18.954' N, 72° 03.588' W). Separate water samples were collected on two separate and consecutive days similar to Chapter 3. The seawater was passed through a 100- μ m mesh similar to Chapter 3. The sieved seawater was sub-sampled for DOC, CHNS, and TSS analysis. The pH, temperature, and salinity were measured and then the water was poured into clean 1-L jars and spiked with 0.25 nM ^{200}HgT standards.

Controls consisted of a marine snow control and a Hg control. The marine snow control consisted of jars left on a stationary surface such that no visible marine snow was produced (i.e., unrolled); for every rolled jar there was one unrolled jar. The Hg control consisted of 6 replicates of unrolled jars that did not have $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT spikes added to the water. Each day had six rolled, spiked jars and six unrolled, spiked jars. All of the jars were kept at 18 °C in an environmental chamber under light/dark cycle.

After four days, visible marine snow was collected from the jars similar to the previous chapters. The remaining liter of water was filtered through an 8- μ m polycarbonate filter. A fraction (60 mL) of the 8- μ m filtrate was filtered through a 0.2- μ m polycarbonate filter. The remaining 8- μ m filtrate was filtered through a pre-ashed QF/F. The final filtrate was saved in clean 250-mL ICHEM bottles for ^{200}HgT ICPMS analysis. A sub-sample of the final filtrate was saved for DOC analysis. All polycarbonate filters were frozen and then freeze dried for 72 hrs. The dried filters were weighed and then acid digested in 7-mL of 4.57M HNO_3 (Trace Metal Grade) in a 60 °C water bath overnight.

A 3-mL aliquot of the 7-mL digest was transferred into clean tubes, diluted up to 12 mL with $\text{MQ-H}_2\text{O}$, spiked with 0.24-mL BrCl , and incubated in a 60 °C bath overnight. The resulting digest was spiked with ^{201}HgT internal standard and hydroxylamine (in excess) and analyzed using the FIAS-ICPMS instrumentation described in Chapter 3. The filtrates were analyzed for ^{200}HgT by spiking the 250-mL

ICHEM jars with 0.5-mL BrCl and left to incubate at room temperature overnight. The oxidized water sample was spiked with internal ^{201}HgT standard and hydroxylamine before FIAS analysis. Incorporation percentages and log K_d values were calculated for each particle fraction using equations 2.1 and 2.2; respectively.

Results and Discussion

Masses were similar in both experiments (unlike the data presented in Chapter 3) illustrating how natural variability can give very different results between studies. Particulate mass, on a per liter basis, was greatest in the 0.2- to 8- μm fraction in both treatments. Marine snow ($>300\ \mu\text{m}$) was the second greatest in the rolled treatment and the 8- to 300- μm fraction was the second greatest in the unrolled treatment. The masses presented in Figure 22 (see below) are on a per volume basis to correct for the volume filtered. Although the 0.2- to 8- μm mass fraction was greatest it is the least bioavailable to the mussels. Unlike the data presented in Chapter 3, there is a clear difference between the mass of the marine snow fraction in the rolled treatment versus the unrolled treatment indicating that there were less large particulates that settled in the unrolled jars. It is interesting to note that the mass of the 8- to 300- μm fraction was higher than expected in the rolled treatment even after the marine snow was collected. The mass of the 8- to 300- μm fraction was significantly greater in the unrolled jars than the rolled jars. This result supports my hypothesis in my previous chapter that there was a large concentration of bioavailable particulates in the unrolled jars that the mussels may have been able to ingest. It also shows that there was still a measureable mass of this fraction available even after marine snow was produced indicating that not all of the particulates were incorporated into the marine snow after four days.

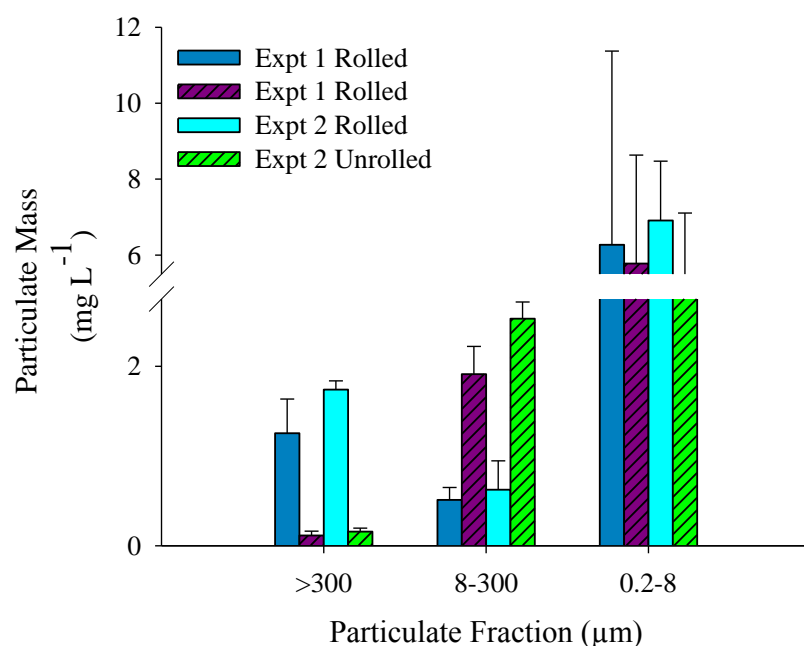


Figure 22- Masses of particulate fractions filtered from 1-L jars on a per volume basis. Data represents the mean \pm SD; n=6.

The ^{200}HgT incorporation results (see below; Figure 23) demonstrate a similar trend to the masses in the marine snow and mid-range size fractions, but a different trend in the smallest size fraction. Incorporation of ^{200}HgT was lowest in the 0.2- to 8-μm fraction in both treatments even though this represented the greatest fraction on a mass per volume basis (see Table I.1). Incorporation in the marine snow was significantly greater in the rolled treatment than the unrolled treatment, unlike my previous results. Again, this is due to natural variability and methodological practices and shows that difference in incorporation across the previous experiments is most likely driven by differences in the mass of marine snow produced.

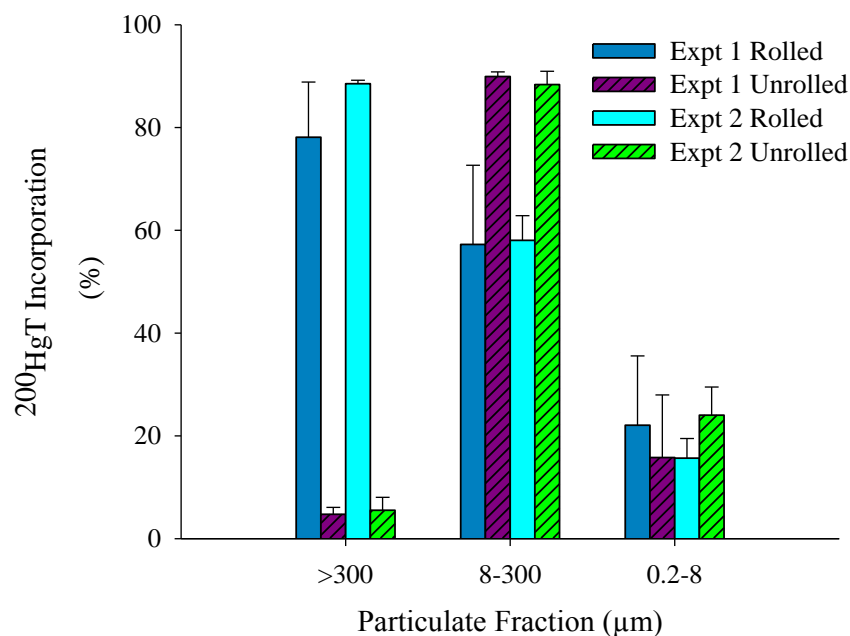


Figure 23- Incorporation of ^{200}HgT into the various particulate fractions collected. Data represent the mean \pm SD; n=6.

The most interesting result is the incorporation values in both the rolled and unrolled treatments for the mid-range size fraction (8 to 300 μm). In the unrolled treatment, the incorporation is comparable to the incorporation into the marine snow in the rolled treatment. In the rolled treatment, the incorporation is significantly higher in comparison to what was incorporated into the marine snow fraction. The high incorporation into the unrolled mid-range particles confirms my hypothesis that the mussels were able to ingest similar proportions of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT from bioavailable particulates in the unrolled treatment. These results explain the similar uptake of HgT from rolled and unrolled treatments in Chapter 2 and the lack of significant difference between unrolled and rolled treatments in Chapter 3.

Partition coefficients were calculated to further analyze the distribution of ^{200}HgT amongst the particle fractions (Table I.1). Two-way ANOVA results indicated a significant interaction affect between the experiment and the particle fraction ($p < 0.05$). There was a significant difference between the rolled and unrolled log K_d values in the $>300\mu\text{m}$ (i.e., marine snow) fraction in both experiments. There was no significant difference in the rolled and unrolled treatments in the 8 to $300\mu\text{m}$ fraction in both

experiments. There was a significant difference between the rolled and unrolled treatment in the smallest fraction in experiment 2, but not in experiment 1. Summarized ANOVAR results are listed in Table I.2. Partition coefficients were similar across all experiments, all treatments, and all size fractions. The highest values were calculated for the 8- μm size fraction indicating that ^{200}HgT partitioned more to the particles smaller than marine snow.

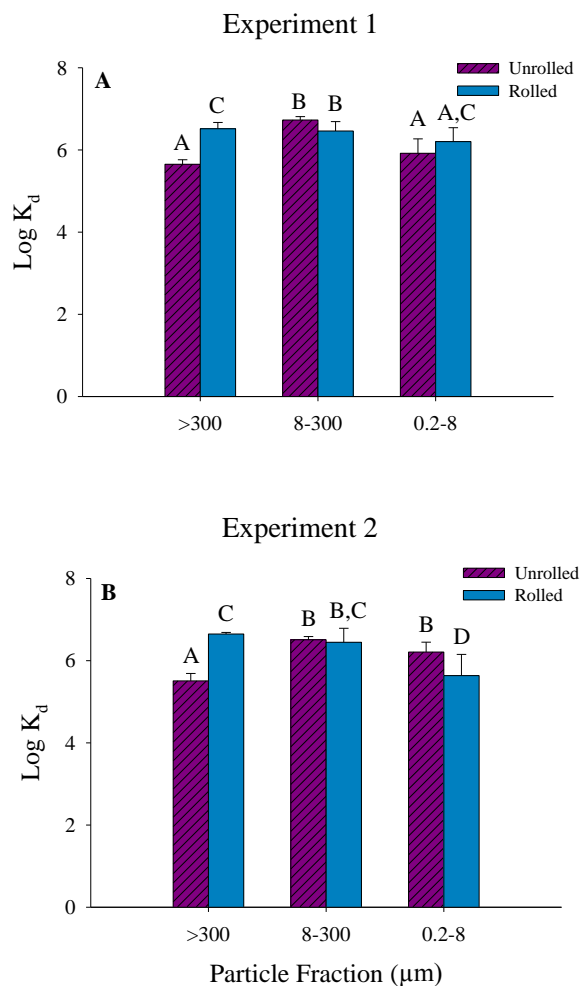


Figure 24-Partition coefficients (Log K_d) for each experiment 1(A) and 2(B). Unrolled treatments are colored in purple and rolled treatments are colored in blue. Each particle size fraction is separated on the y-axis into >300 μm , 8-300 μm , and 0.2-8 μm . Two-way repeated measures (ANOVAR) indicated a significant interaction ($p < 0.01$) between treatment and particle fraction. Letters above the bars indicate significant difference. Those that share a similar letter are not significantly different; those with different letters are significantly different.

This result is contrary to what I anticipated. I expected the highest partitioning to occur in the marine snow as the marine snow entrained and coagulated more organic matter as it formed. I suggest that the partitioning was greatest in the mid-sized fraction because there was more surface to area for the Hg to bind to before the particulates were incorporated into marine snow. But, in counter argument to this suggestion, the K_d of the smallest fraction is less than the 8- μ m fraction. This would negate the surface area argument. The final suggestion to make is that the K_d was lower for the smallest fraction because there was so little left in suspension. It is important to note that upon calculating a mass balance for this experiment, the total of the masses collected did not equal the total TSS at the beginning of the experiment. This discrepancy may affect the log K_d values because the partitioning depends on the suspended particles available as binding sites. It may be that the TSS and, subsequently, the log K_d values were affected by the presence of plankton remaining in the water after sieving. These plankton may not only be accumulating HgT thereby reducing the labile HgT in solution, but they were likely ingesting particulates and respiring during the rolling period. The degradation of the organic materials by the plankton as they respire may change the partitioning and the particulate composition during the rolling period. Nonetheless, with the relatively high incorporation and the highest partition coefficients in the 8 μ m fraction, it would appear that the particles smaller than marine snow are where most of the Hg is bound, again reinforcing the final conclusion of Chapter 2 and Chapter 3 that the role of smaller particulates appears to be more important than the marine snow. In future experimentation, the only way to account for these methodological complications would be to filter the water such that the mussels would only feed on specific size fractions.

Table I.1-Spike concentrations, percent incorporation values, partition coefficients, TSS, and DOC concentrations for all treatments and experiments. TSS was taken after the water was sieved. The DOC reported is the average DOC for each treatment after all size fractions were filtered.

Parameter	²⁰⁰ HgT			
	Experiment 1		Experiment 2	
	Unrolled	Rolled	Unrolled	Rolled
Spike Concentration (nM)	0.12		0.12	
Aggregate Incorporation (%)	4.74 ± 1.4	78.1 ± 10.7	5.5 ± 2.5	88.5 ± 0.66
8µm Incorporation (%)	89.9 ± 0.90	57.2 ± 15.4	88.4 ± 2.6	58.0 ± 4.8
0.2µm Incorporation (%)	15.8 ± 12.2	22.1 ± 13.5	24.0 ± 5.5	15.7 ± 3.8
Aggregate Log Kd	5.6 ± 0.1	6.5 ± 0.1	5.5 ± 0.2	6.6 ± 0.04
8µm Log Kd	6.7 ± 0.1	6.5 ± 0.2	6.5 ± 0.1	6.4 ± 0.3
0.2µm Log Kd	5.9 ± 0.35	6.2 ± 0.3	6.2 ± 0.2	5.6 ± 0.5
TSS (mg L ⁻¹) [§]	14.87 ± 3.0		9.9 ± 0.4	
DOC (µM) [¥]	302.4 ± 7.6	295.3 ± 30.0	363.9 ± 13.7	367.4 ± 21.7

Values represent the means ± SD; n=6

§TSS collected on each experimental day before rolling

¥Average DOC for each treatment after aggregate collection

*Collected mass=0

Table I.2-Two-way ANOVAR results table for data presented in Figure 24. Any p value <0.05 is considered significant.

Fraction Incorporations				
Source	df	MS	F	p
Experiment 1				
Treatment	1	0.718	13.652	0.0040
1-L Jar	10	0.5260		
Fraction	2	2.1590	20.293	<0.001
Treatment x Fraction	2	1.9230	18.070	<0.001
Residual	19	1.0110		
Total	34	6.423		
Experiment 2				
Treatment	1	0.257	3.546	0.089
1-L Jar	10	0.072		
Fraction	2	0.989	11.769	<0.001
Treatment x Fraction	2	2.320	27.612	<0.001
Residual	20	0.084		
Total	35	0.265		

Conclusions

In conclusion, my hypothesis that ^{200}HgT was incorporated, at a high percentage, into smaller particle fractions than marine snow was proved correct. Incorporation of ^{200}HgT was significantly greater in the marine snow fraction in the rolled treatment, but significantly greater in the 8- to 300- μm fraction in the unrolled treatment. Therefore, it is likely that the mussels exposed to contaminated-marine snow in the previous chapters were ingesting both forms of Hg from other particulates than marine snow. Additionally, there was enough incorporation into remaining particulates ranging from 8 to 300 μm in the rolled treatments that the mussels could select for those particulates over the marine snow. These results explain why ingestion, absorption, and accumulation were independent of the amount of ^{200}HgT incorporated into marine snow. Again, I have added more evidence to support that idea that marine snow is not an important pathway of Hg uptake into blue mussels, but rather the smaller particles are more important.

Appendix II-Data Summaries

Chapter 2-Supplemental Data

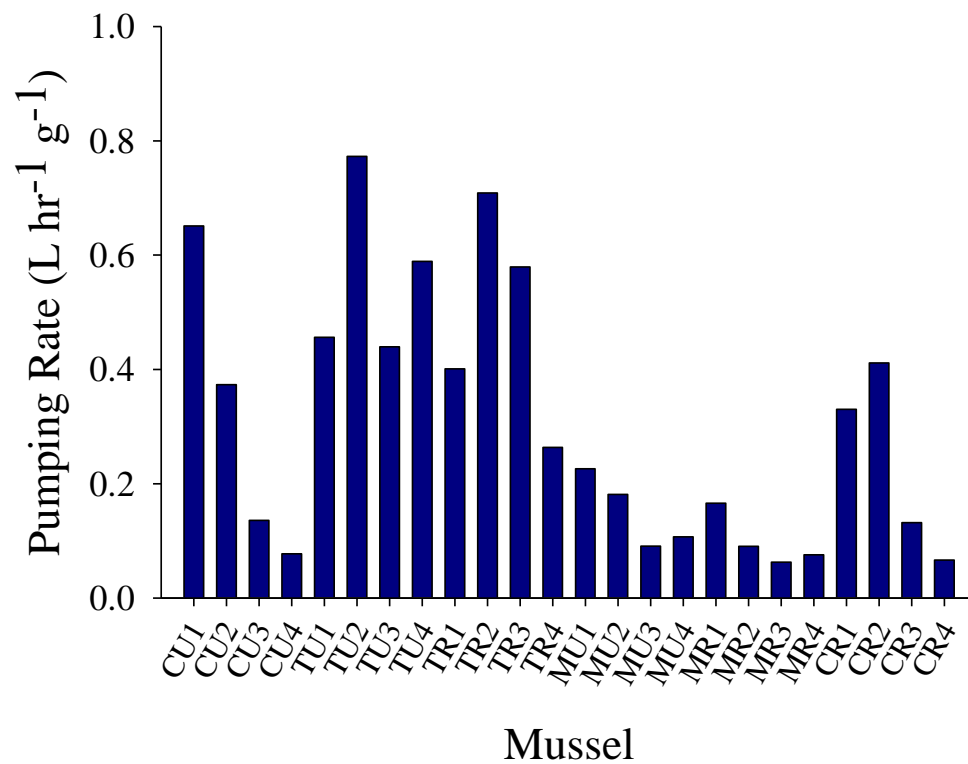


Figure 25- Pumping rate for each animal based upon bead counts from fecal pellet and digestive gland digests. Pumping rates were standardized to a standard mussel rate (equation 2.4). C represents the controls, T represents animals exposed to HgT, and M represent animals exposed to CH₃Hg. U represents unrolled treatments and R represents rolled treatments.

Chapter 2-Statistical Summary

Table II.1-Summary Tables of two-way ANOVAR (A) and two-way ANOVA (B, C) analyses of data presented in Chapter 2.

(A) Tissue Concentrations				
Source	df	MS	<i>F</i>	p
¹⁹⁹ CH ₃ Hg				
Treatment	1	0.0557	3.478	0.1110
Animal (treatment)	6	0.0160		
Tissue	3	0.0262	8.293	0.0010
Treatment x Tissue	3	0.2930	9.275	<0.001
Residual	18	0.0032		
Total	31	0.0121		
²⁰⁰ HgT				
Treatment	1	2.927	3.421	0.114
Animal (treatment)	6	0.856		
Tissue	3	4.049	7.886	0.001
Treatment x Tissue	3	1.281	2.494	0.093
Residual	18	0.513		
Total	31	1.074		
(B) Body Burden Concentrations Normalized to Feeding Rate				
Source	df	MS	<i>F</i>	p
Treatment	1	0.0045	0.0043	0.949
Spike	1	2.3620	2.2340	0.161
Treatment x Spike	1	12.831	12.137	0.005
Residual	12	1.0570		
Total	15	1.8590		
(C) Feces Concentration Normalized to Feeding Rate				
Source	df	MS	<i>F</i>	p
Treatment	1	100.165	0.0070	0.935
Spike	1	891859	62.260	<0.001
Treatment x Spike	1	244.58	0.0171	0.898
Residual	12	14324.7		
Total	15	70940.0		

Chapter 3-Supplemental Data

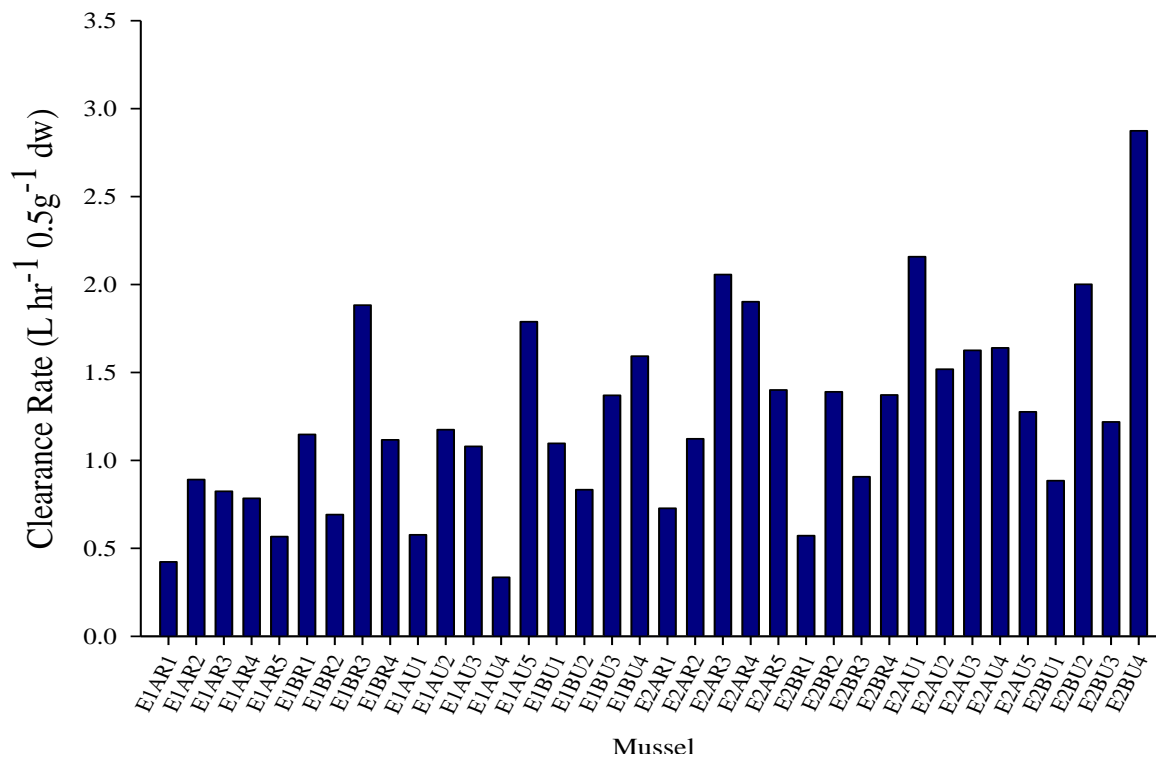


Figure 26-Calculated clearance rates standardized to a standard weight of 0.5g for mussels exposed to ¹⁹⁹CH₃Hg and ²⁰⁰HgT. E stands for experiment and the following number represents the experiment number. A or B represent the different groups fed on each experimental day. There was only enough room for 12 mussels on the stir plates per 2-hr feeding period so there were two groups of 12 mussels fed on each day. R and U represent the treatment the mussel (i.e., rolled and unrolled). The final number represents the replicate number for each feeding group and each treatment.

Table II.1- Summary of results including calculated incorporations, log K_d, gross ingestion efficiencies, absorption efficiencies, residual accumulations, TSS, and DOC concentrations. Values represent the means ± SD.

	¹⁹⁹ CH ₃ Hg				²⁰⁰ HgT			
Parameter	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	Unrolled	Rolled	Unrolled	Rolled	Unrolled	Rolled	Unrolled	Rolled
Spike Concentration (nM)	0.24		0.22		0.24		0.22	
Incorporation (%)	20.57 ± 2.65	27.80 ± 3.51	1.51 ± 0.79	5.84 ± 1.15	8.46 ± 2.57	16.99 ± 2.89	0.31 ± 0.22	1.56 ± 0.46
Log K _d [§]	4.69 ± 0.09	4.82 ± 0.05	4.69 ± 0.05	4.95 ± 0.04	4.36 ± 0.10	4.54 ± 0.02	3.97 ± 0.06	4.28 ± 0.09
Gross Ingestion Efficiency	5 ± 3	6 ± 2	14 ± 4	11 ± 8	13 ± 6	19 ± 3	26 ± 5	19 ± 1
Absorption Efficiency (%)	0.5°	0.8 ± 0.8	15°	12°	0.7°	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.2
36hr RA* (%)	0.4°	0.4°	5°	11°	1°	0.2°	0.2°	0.2°
72hr RA (%)	0.4°	23°	6°	54°	0.4°	0.2°	0.3°	0.1°
TSS (mg/L)	26.43 ± 2.45		20.4 ± 7.4		26.43 ± 2.45		20.4 ± 7.4	
DOC (µM)	173.54 ± 5.30		144.91 ± 3.10		173.54 ± 5.30		144.91 ± 3.10	

Values represent the means ± SD

*Residual Accumulation (RA) is a similar Assimilation Efficiency, but does not account for excretion. Error represents the range.

°Low sample number, no standard deviation

[§]Log K_d values less than published values (Stordal et al, 1996)

Chapter 3-Statistical Summary

Table II.2-Two-way ANOVA summary tables of $^{199}\text{CH}_3\text{Hg}$ and ^{200}HgT incorporated into marine snow (A) and marine snow mass (B)

(A) Incorporation into Marine Snow				
Source	df	MS	<i>F</i>	p
$^{199}\text{CH}_3\text{Hg}$				
Experiment	1	1009.8	173.75	<0.001
Treatment	1	80.161	13.793	0.0100
Experiment x Treatment	1	5.0560	0.8700	0.3870
Residual	6	5.8120		
Total	9	133.55		
^{200}HgT				
Experiment	1	370.83	85.240	<0.001
Treatment	1	63.670	14.630	0.0060
Experiment x Treatment	1	35.340	8.1200	0.0250
Residual	7	4.3500		
Total	10	51.270		
(B) Marine Snow Mass				
Source	df	MS	<i>F</i>	p
Experiment	1	57.01	1341.65	<0.001
Treatment	1	0.670	15.2900	0.0080
Experiment x Treatment	1	0.001	0.15000	0.7100
Residual	6	0.040		
Total	9	6.960		
*Low mass outlier removed from data				

Table II.3- Statistical summary of linear regression analyses of $^{199}\text{CH}_3\text{Hg}$ data from Chapter 3. Table includes descriptive statistics, correlation coefficients, and p-values. No transformations were required for these data, the analysis of the homogeneity of the variances passed.

Parameter	$^{199}\text{CH}_3\text{Hg}$						
	n	Mean	SD	SE	r^2	p value	Figure
Gross Ingestion vs Incorporation	12	8.90	5.80	1.70	0.32	0.07	17A
Ingestion (as available in flocs) vs Incorporation	12	5.40	7.90	2.30	0.20	0.20	18A
Absorption Efficiency vs Incorporation	12	29.82	42.77	12.35	0.22	0.15	19A
Residual Accumulation 36hr vs Incorporation	8	4.32	5.89	2.07	0.35	0.12	20A
n refers to the sample number							
SD refers to the standard deviation							
SE refers to the standard error							
r^2 refers to the correlation coefficient of the regression							

Table II.4- Statistical summary of linear regression analyses of ^{200}HgT data from Chapter 3. Table includes descriptive statistics, correlation coefficients, and p-values. Data that failed the homogeneity of variance test were log transformed and F-tested using Table B.4 in Zarr, 1984.

Parameter	^{200}HgT					p value	Figure	Transformation	Re-test
	n	Mean	SD	SE	r^2				
Gross Ingestion vs Incorporation	12	19.40	6.30	1.80	0.08	0.40	17B	NA	NA
Ingestion (as available in flocs) vs Incorporation	12	27.10	41.90	12.10	0.30	0.06	18B	Log10	$r^2=0.68$ / $p=0.002$
Absorption Efficiency vs Incorporation	12	8.71	28.75	8.30	0.02	0.71	19B	Log10	$r^2=0.01$ / $p=0.76$
Residual Accumulation 36hr vs Incorporation	8	23.35	8.94	3.16	0.09	0.45	20B	NA	NA
n refers to the sample number SD refers to the standard deviation SE refers to the standard error r^2 refers to the correlation coefficient of the regression									